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Leishmania major: clathrin and adaptin complexes of an intra-cellular parasite

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Abstract

To investigate the role of clathrin-mediated trafficking during the *Leishmania* lifecycle, open reading frames encoding clathrin heavy chain and the β-adaptins, major components of the adaptor complexes, have been analysed both *in silico* and experimentally. The *Leishmania* genome encodes three β-adaptins, which arose at a time predating speciation of these divergent trypanosomatids. Unlike *Trypanosoma brucei*, both clathrin heavy chain and β-adaptin1 are constitutively expressed throughout the *Leishmania* life cycle. Clathrin relocalises in amastigotes relative to promastigotes, consistent with developmental alterations to the morphology of the endo-membrane system.
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Index Descriptors: Leishmania; Kinetoplastid; Intra-macrophage parasite; Clathrin; Adaptin; Evolution; Trafficking

Clathrin forms the coat protein complex central to much of vesicular trafficking at the plasma membrane (endocytosis) and from the *trans*-Golgi network (TGN) directed towards endosomal/lysosomal compartments (Kirchhausen, 2000). A vast number of accessory proteins associate with clathrin in a coordinated manner, the most abundant of which are the heterotetrameric adaptor protein (AP) complexes involved in cargo recruitment (Robinson, 2004). Four closely related AP complexes have been identified in humans: AP-1 functions in TGN-endosome transport; AP-2 in plasma membrane-endosome trafficking; AP-3 is found near endosomes (Dell'Angelica et al., 1997; Simpson et al., 1997); and AP-4 close to the TGN (Dell'Angelica et al., 1999; Hirst et al., 1999). Each complex is composed of two large subunits, a medium subunit and a small subunit. In *Saccharomyces cerevisiae*, only AP-1, -2, and -3 are present (Robinson, 2004).

Structures resembling clathrin coated pits and vesicles have been identified in *Leishmania* by ultra-structural studies (Weise et al., 2000). In addition, a recent study demonstrated that clathrin/adaptin-mediated trafficking (specifically involving AP-1) is essential for intra-macrophage survival in *Leishmania* spp. (Gokool, 2003). To enable further analysis, the full-length open reading frames for clathrin heavy chain (LmCHC) and the three β-adaptins (LmBAPs) were retrieved from the complete *Leishmania major* genome (http://www.genedb.org). The levels of identity/similarity to the closest human and *Trypanosoma brucei* homologues are shown in Table 1. The large β-subunits of the adaptor complexes were chosen for analysis due to the availability of a cross-reacting antibody (Morgan et al., 2001) and their utility in defining distinct adaptor complexes. Phylogenetic analysis of the adaptin amino acid sequences (Fig. 1) indicated that

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Table 1
Sequence identity and similarity for *Leishmania* clathrin heavy chain (LmCHC) and β-adaptin (LmBAP) subunits (GeneDB accession numbers in parentheses)

	T. brucei	*H. sapiens*	Orthologue
LmCHC (LmjF36.1630)	63 (79)	38 (60)	CHC
LmBAPa (LmjF36.6770)	58 (63)	35 (57)	β1
LmBAPb(LmjF36.5260)	41 (59)	27 (42)	β3
LmBAPc[a](LmjF11.0990)	29 (47)	29 (47)	β1/β2

In each case, the percentage identity and similarity (in parentheses) are shown for the *Leishmania* hypothetical translations against the sequence orthologues from African trypanosomes and humans.

[a] Closest orthologue in *T. brucei* is β-adaptin1 and in humans β-adaptin2.

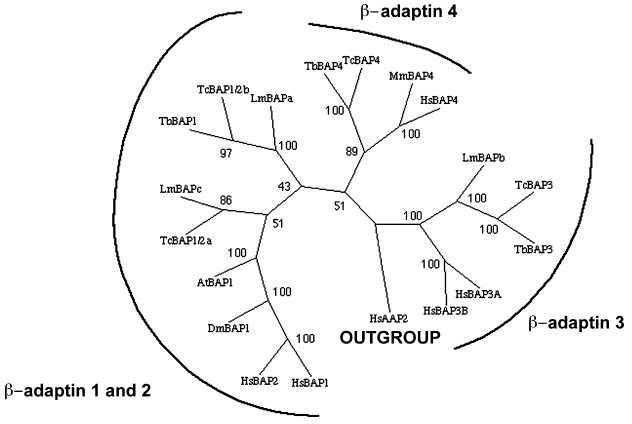

Fig. 1. Phylogenetic tree generated after maximum parsimony analysis of β-adaptins (BAPs). Sequence alignments were done using ClustalW (Jeanmougin et al., 1998). Phylogenetic analyses were performed on an edited alignment of the conserved amino-terminal 600 amino acids, the trunk region, of the β-adaptins (Schledzewski et al., 1999) using maximum parsimony (Protpars, Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c; distributed by the author, Department of Genetics, University of Washington, Seattle, USA). Bootstrap values from 100 replicates are indicated. Human α-adaptin2 (HsAAP2) was utilised as an outgroup. Lm—*L. major*: LmBAPa (GeneDB LmjF36.6770); LmBAPb (GeneDB LmjF36.5260); LmBAPc (GeneDB LmjF11.0990); Tb—*T. brucei*: TbBAP1 (GeneDB Tb10.6k15.2500); TbBAP3 (GeneDB Tb11.01.2420); TbBAP4 (GeneDB TRYPtp6k15-3d06.p1c_172); Tc—*T. cruzi*: TcBAP1/2a (GeneDB Tc00.1047053510101.15000); TcBAP1/2b (GeneDB Tc00.1047053506247.200); TcBAP3 (GeneDB Tc00.1047053506673.60); TcBAP4 (GeneDB Tc00.1047053504137.60); Hs—*Homo sapiens*: HsBAP1 (SWALL Q10567); HsBAP2 (SWALL P21851); HsBAP3A (SWALL O00203); HsBAP3B (SWALL Q13367); HsBAP4 (SWALL Q9Y6B7); At—*Arabidopsis thaliana*: AtBAP1 (SWALL Q9SUS3); Dm—*Drosophila melanogaster*: DmBAP1 (SWALL Q24253); Ce-*Caenorhabditis elegans*: CeBAP3 (SWALL O45718); Mm—*Mus musculus*: MmBAP4 (SWALL Q9WV76); outgroup: HsAAP2 (SWALL O95782).

LmBAPa and LmBAPc form part of AP-1/2 complexes; due to high sequence conservation, AP-1 and AP-2 β-adaptins are indistinguishable at this level (Schledzewski et al., 1999). LmBAPb is likely to be an AP-3 β-adaptin, LmBAP3. Therefore, like the related protozoan *T. brucei* (and the yeast *S. cerevisiae*), *Leishmania* are only capable of assembling three adaptor complexes, but whereas *T. brucei* lacks AP-2, it is likely that AP-4 is the absent complex in *Leishmania*. This interpretation is supported by the presence in *L. major* genome database of the other subunits required for AP-1, AP-2, and AP-3, but a lack of those required for AP-4 (data not shown). *Leishmania* therefore resembles *S. cerevisiae* in its complement of adaptor complexes.

In addition to providing a preliminary classification of the *Leishmania* β-adaptins, this analysis also indicates that the formation of the adaptor complexes occurred prior to the speciation of trypanosomatids approximately three billion years ago, making them an ancient feature of the eukaryotes. It is notable that another trypanosomatid, the intra-cellular parasite *Trypanosoma cruzi*, possesses a full complement of four β-adaptins (Fig. 1) and the other subunits required (data not shown) to form four adaptor complexes. Thus, the lack of LmAP-4 and TbAP-2 is probably due to secondary loss of the genes encoding the subunits (e.g., β-adaptins) required to form these complexes. The reasons for the differences in the complement of adaptins in these closely related parasites remain unclear, although it has been proposed that by dispensing with AP-2 (the adaptor complex that functions in clathrin-mediated endocytosis), the *T. brucei* endocytic system may become streamlined to ensure the rapid, but relatively non-selective, uptake of the highly abundant variant surface glycoprotein (VSG), reactive antibody, and other components (Allen et al., 2003). The role of AP-4 in other systems has yet to be clearly elucidated (Robinson, 2004), so the functional significance of the loss of AP-4 in both *S. cerevisiae* and *L. major* remains unclear. In contrast, perhaps the higher level of complexity (a full complement of four AP complexes) in *T. cruzi* is essential for an intra-cellular parasite that can inhabit a range of host cell types, an adaptation that requires modulation of parasite surface molecules and active escape from the parasitophorous vacuole into the cytoplasm (Tan and Andrews, 2002).

Leishmania pathogenic amastigotes endocytose and degrade MHC class II molecules and the class II co-factor H-2M in infected mammalian macrophages (Antoine et al., 1998, 1999), observations that have led to the proposal that antigen presentation and parasite killing may be modulated via the parasite's endocytic system. Significantly, proteolytic activity is highly upregulated as *Leishmania* differentiate from insect stage promastigotes into amastigotes, correlating with the appearance of multi-vesicular lysosomes (megasomes) and perhaps increased endocytic activity (Courret et al., 2001). These morphological and physiological changes in the endomembrane system may reflect functional differences between the insect (promastigote) and mammalian (amastigote) stages of the parasite. Although such differences could play a role in pathogenesis, there are no reported experimental data that would support the conclusion that endocytosis and intra-cellular trafficking per

se is upregulated in amastigotes. To address this issue, we have begun an experimental characterisation of clathrin heavy chain (LmCHC) and a β-adaptin (LmBAPa) with a view to understanding the components of the endo-membrane trafficking machinery and their potential role in pathogenesis.

We examined the expression of LmCHC and LmBAPa during the parasite life cycle using cross-reacting antibodies (Morgan et al., 2001). Unlike in *T. brucei* pathogenic bloodstream forms (Morgan et al., 2001), there is no significant upregulation of clathrin heavy chain or β-adaptin expression in any of the examined life

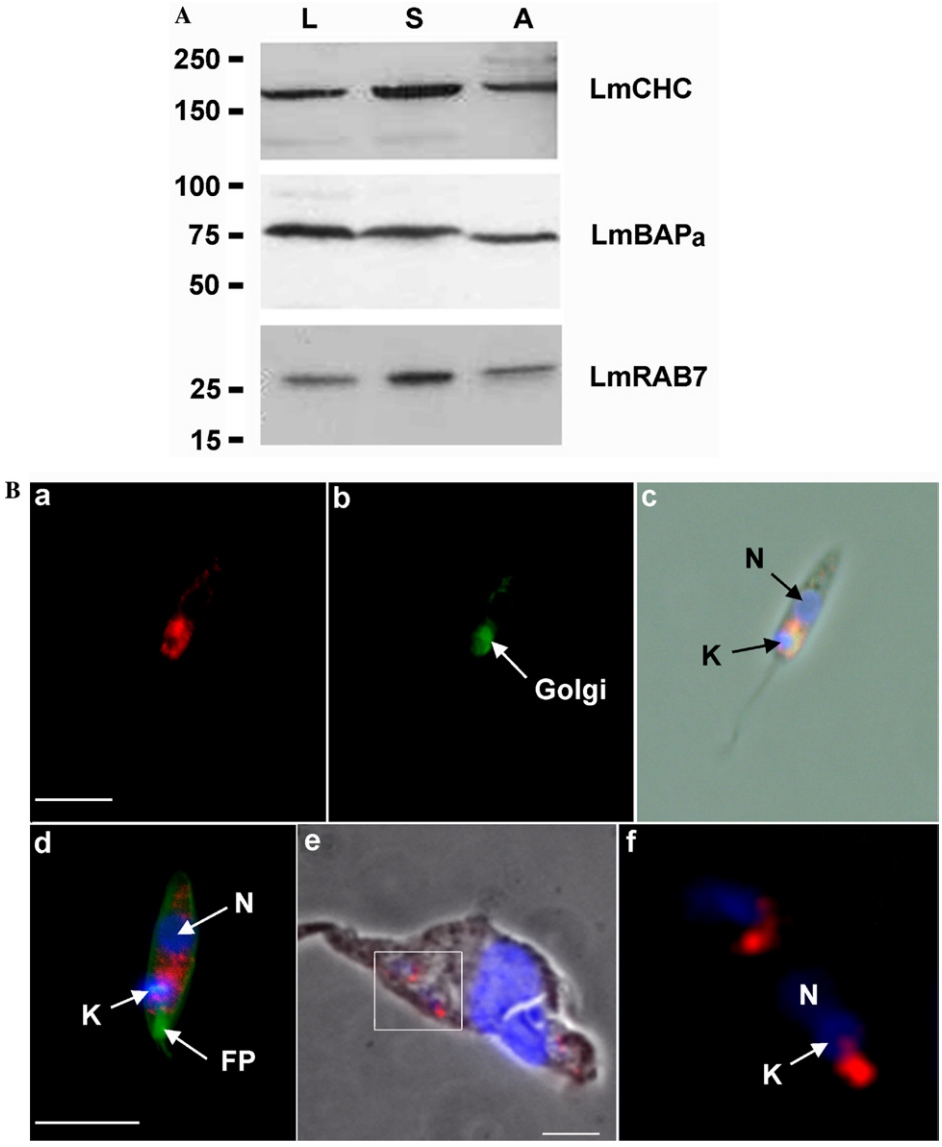

Fig. 2. *Leishmania major* (MHOM/IL/81/Friedlin) promastigotes were maintained and used to infect peritoneal macrophages from BALB/c mice as described (Denny et al., 2002). Western analyses and immuno-fluorescence assays were carried out as previously described (Denny et al., 2002) using cross-reacting *T. brucei* antibodies raised against TbCHC and TbBAP1 (an anti-peptide antibody with a conserved region in LmBAPa only) (Morgan et al., 2001) at 1:1000 (Western) and 1:100 (immuno-fluorescence) , respectively. (A) Western analyses of clathrin heavy chain (LmCHC) and a β-adaptin (LmBAPa) expression levels through the *Leishmania* life cycle. Protein bands were detected close to the predicted molecular weights of LmCHC (190 kDa) and LmBAPa (82 kDa). Both polypeptides are constitutively expressed throughout the life cycle of *L. major* (L, logarithmic phase procyclic promastigotes; S, stationary phase metacyclic-enriched promastigotes; and A, lesion amastigotes). LmRAB7 was used as a loading control (Denny et al., 2002). The positions of relevant molecular weight markers (kDa) are indicated on the left. (B) Immuno-localisation of β-adaptin1 (LmBAP1) and clathrin heavy chain (LmCHC) in *L. major*. *Leishmania* promastigote: a, LmBAPa (red); b, Golgi apparatus visualised using a mutant 18HASPB::GFP fusion protein (Denny et al., 2000) (green); c, a and b merged and overlayed with DAPI stain (blue) and a phase image. LmBAPa partially co-localises with the Golgi (yellow in c) verifying it as LmBAP1. Scale bar: 5 μm. d, *Leishmania* promastigote. LmCHC (red); plasma membrane/flagellar pocket visualised using a GFP fusion protein [18HASPB:: GFP (Denny et al., 2000); green]; DAPI (blue). Scale bar: 5 μm. e, Intra-macrophage *Leishmania* amastigote. LmCHC (red); DAPI (blue); overlayed with a phase image. Scale bar: 10 μm. and f, Fourfold magnification of area highlighted in e without phase image. N, nucleus; K, kinetoplast; and FP, flagellar pocket.

cycle stages (Fig. 2A), indicating that clathrin and AP-1 do not directly mediate any quantitative increase in endocytic/endosomal trafficking in intra-macrophage amastigotes. In addition to changes in expression levels, differential localisation of TbCHC has been observed during the *T. brucei* life cycle (Morgan et al., 2001).

We also used the cross-reacting antibodies to localise LmCHC and LmBAPa in different stages. In *Leishmania* promastigotes, LmBAPa partially co-localised with the Golgi apparatus but was also present on structures throughout the cell (Fig. 2B, a–c), a distribution similar to that of TbBAP1 in *T. brucei* (Morgan et al., 2001). This observation indicated that LmBAPa is very likely to be an AP-1 β-adaptin (LmBAP1) and hence a true functional orthologue of TbBAP1. Cross-reactivity with mammalian epitopes prevented the acquisition of clear images of LmBAP1 in intra-macrophage amastigotes using anti-TbBAP1 antibody. In *Leishmania* promastigotes, LmCHC was localised to vesicular structures (Fig. 2B, d) distributed throughout the cell, although there was a concentration of the protein towards the apical end of the parasite near to the kinetoplast (the mitochondrial genome) and the Golgi. Some staining was observed very near to the flagellar pocket, the sole site of exo- and endocytosis, possibly correlating with coated pits (Weise et al., 2000). As described above, clathrin is not just involved in mediating endocytosis from the plasma membrane but also functions, with AP-1, in TGN-endosomal/lysosomal transport. The lysosome-like multi-vesicular tubule (MVT) of *Leishmania* promastigotes stretches between the apical and basal ends of the parasite (Ghedin et al., 2001; Mullin et al., 2001; Weise et al., 2000). The extensive nature of this organelle could account for the wide spread distribution of LmBAP1 as well as LmCHC.

Similar analyses of intra-macrophage amastigotes localised LmCHC to a distinct body close to the kinetoplast (Fig. 2B, e and f). This redistribution between parasite stages is comparable to that observed for LmRAB7 (Denny et al., 2002) and probably reflects the morphological changes in the endo-membrane system that occur during the parasitic life cycle, when the promastigote MVT is replaced by the highly lytically active megasomal compartments. These structural alterations may reflect changes in endocytic activity caused by the requirement of intra-macrophage amastigotes to endocytose and degrade host factors to avoid immune detection.

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