The AP3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*

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Summary

Lysosomal function is crucial for the differentiation and infectivity of the parasitic protozoon *Leishmania major*. To study lysosomal biogenesis, an *L. major* mutant deficient in the δ subunit of the adaptor protein 3 (AP3 δ) complex was generated. Structure and proteolytic capacity of the lysosomal compartment were apparently unaffected in the AP3-deficient mutant; however, defects were identified in its acidocalcisomes. These are acidic organelles enriched in calcium and phosphorus, conserved from bacteria to eukaryotes, whose function remains enigmatic. The acidocalcisomes of the *L. major* mutant lacked membrane-bound proton pumps (notably V-H$^+$-PPase), were less acidic than normal acidocalcisomes and devoid of polyphosphate, but contained a soluble pyrophosphatase. The mutant parasites were viable in vitro, but were unable to establish an infection in mice, which indicates a role for AP3 in determining – possibly through an acidocalcisome-related function – the virulence of the parasite. AP3 transport function has been linked previously to lysosome-related organelles such as platelet dense granules, which appear to share several features with acidocalcisomes. Our findings, implicating that AP3 has a role in transport to acidocalcisomes, thus provide further evidence that biogenesis of acidocalcisomes resembles that of lysosome-related organelles, and that both may have conserved origins.

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Key words: Adaptor protein, AP3, Acidocalcisomes, Membrane-protein transport, *Leishmania*

Introduction

Lysosomes are cytoplasmic organelles that function as major degradative sites within eukaryotic cells. Since the pioneering work of de Duve and co-workers that led to the naming of the organelles (de Duve et al., 1955), lysosomes are recognised as being more than membrane-bound compartments full of hydrolases, and a number of related organelles with very specialised functions have been identified. For example, lysosomes are the final intracellular destination for soluble proteins internalised by endocytosis, however, many of the characteristics and features of lysosomes are shared with other related compartments, the cellular functions of which are diverse (Dell'Angelica et al., 2000). Indeed, certain cell types contain lysosome-related organelles whose roles include synthesis and storage of pigments (melanosomes in higher eukaryotes and pigment granules in insects), killing of tumour and virus-infected cells (the lytic granules of cytotoxic T lymphocytes and natural killer cells), and activation of platelet aggregation (platelet dense granules). Whereas some of the lysosome-related organelles are only the modified lysosome of a particular cell type, other examples – such as melanosomes or platelet dense granules – have a highly specialised function and co-exist with conventional lysosomes in the same cell (Dell'Angelica et al., 2000). The biogenesis of both the lysosomal (Luzio et al., 2003) and lysosome-related (Starcevic et al., 2002) compartments was found to be linked to the secretory and endocytic pathways, but is still not fully understood at the molecular level and subject to controversy. However, a number of naturally occurring mutations and genetic disorders, notably in *Drosophila melanogaster* and mammals, helped characterise several important genes for transport to the lysosome-related compartments, such as the components of the adaptor protein complex 3 (AP3).

AP complexes are key mediators for vesicular transport of membrane proteins between cellular compartments, such as the Golgi complex, endosomes, lysosomes and plasma membrane (for reviews see Boehm and Bonifacino, 2002; Nakatsu and Ohno, 2003). These protein complexes are heterotetramers that coat transport vesicles and comprise two large subunits of ~100-150 kDa (β and α, δ, γ or ε, depending on the complex), one medium subunit (μ) of ~50 kDa and one small subunit (σ) of ~20 kDa. The two large subunits mediate the vesicle-association of the complex by binding to the target membrane and/or clathrin. The medium-sized subunit is involved in specific cargo recognition and the small one is thought to have a role in the stabilisation of the complex. Four different AP complexes have been identified. They are all composed of four specific subunits, each of which is essential, that are not functionally interchangeable between the complexes. Not all complexes are ubiquitously expressed in eukaryotic cells and they have specialised transport functions. For instance, AP1 is involved in post-Golgi sorting to the endosomal compartments, whereas AP2 localises to the plasma membrane and transports proteins to endosomal compartments. The function of the AP4 complex remains obscure, but some data suggest it has a role in sorting to the endosomal-lysosomal system. Involvement of AP3 in sorting of proteins to lysosomes and lysosome-related organelles from the Golgi has been

suggested (Ihrke et al., 2004); other studies propose a role in sorting from endosomes (Peden et al., 2004; Theos et al., 2005).

Mammalian and yeast AP3 complexes were initially recognised as mediators of protein transport to the lysosomes (Cowles et al., 1997; Dell'Angelica et al., 1997). Deficiencies of the δ subunit of AP3 were later also found to lead to pigment-granule deficiencies in *D. melanogaster* (Ooi et al., 1997), suggesting that the function of the complex extends to lysosome-related organelles. The involvement of AP3 in the biogenesis of lysosome-related organelles was further emphasised by the finding that two mouse coat-colour mutants, mocha and pearl, carry mutations in the genes encoding the δ and β3A subunits of AP3, respectively. The mice exhibit hypo-pigmentation of the coat and eyes, prolonged bleeding, and lysosomal abnormalities. These phenotypic characteristics arise from defects in the biogenesis of melanosomes, platelet dense bodies and lysosomes, respectively (Kantheti et al., 1998). AP3 is also present in neuronal tissues, where the two neuron-specific β and μ subunits are expressed. AP3 is involved in transport of Zn^{2+} and Cl^{-} ion channels to the synaptic vesicles of neuronal cells, which is important for synaptic vesicle formation and exocytosis (Salazar et al., 2004a; Salazar et al., 2004b). In mammals, AP3 thus appears to have a number of functions in different cell types, ranging from the biogenesis of lysosome-related organelles in non-neuronal cells to the regulation of exocytic organelles in neurons.

Leishmania major is a flagellated protozoan parasite that causes cutaneous leishmaniasis in humans (Murray et al., 2005). During its life cycle, the parasite alternates between the phlebotomine sandfly and mammalian hosts, and has several developmental forms. Noticeably, the procyclic promastigote is a motile, flagellated form that multiplies in the gut of the fly; the metacyclic promastigote is a non-dividing infective form that resides in the mouthparts of the fly; and the amastigote is a non-motile form that lives and replicates in a parasitophorous vacuole within mammalian macrophages. Parasite survival in these very diverse environments requires exchanges and interactions with the external milieu. To this end, *Leishmania* has a whole network of membranous compartments involved in exocytosis or endocytosis that occur exclusively in a specialised invagination of the plasma membrane termed the flagellar pocket (Landfear and Ignatushchenko, 2001). The terminal stage of the endocytic pathway is the lysosomal compartment, which is rather unusual in promastigote forms of *Leishmania*. It has a very characteristic tubular shape, thus it is named the multivesicular tubule (MVT)-lysosome (Ghedin et al., 2001; Mullin et al., 2001; Weise et al., 2000). This compartment appears to have a low lytic capacity and a relatively high luminal pH in procyclic promastigotes, but acquires the properties of mature lysosomes as the parasite differentiates to the infectious metacyclic form (Mullin et al., 2001). The lysosomal compartment is crucial for development of the infectious stage of the parasite and it is the location of an important virulence factor, the cysteine peptidase CPB (Mottram et al., 2004).

Another major organelle present in *Leishmania* is the acidocalcisome. This was originally defined as an acidic compartment that imported Ca^{2+} by the action of a Ca^{2+}-ATPase (Docampo et al., 1995; Vercesi et al., 1994). Although initially characterised in trypanosomatid species, this organelle has since been identified in other eukaryotes such as the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum*, as well as *Chlamydomonas reinhardtii*, *Dictyostelium discoideum*, and also in several species of bacteria [see Docampo et al. (Docampo et al., 2005) and references therein]. Thus, the acidocalcisome is the only known organelle to be conserved between bacteria and eukaryotes. Furthermore, as there are striking similarities between human platelet dense granules and acidocalcisomes, Ruiz and co-workers have suggested that they may also be conserved in humans (Ruiz et al., 2004).

In this study, we produced a *L. major* AP3δ-null mutant in order to investigate lysosome function. Whereas the null mutants apparently retained lysosomal function, the acidocalcisomes lacked several membrane proteins and were devoid of their usual polyphosphate (polyP) and proton content – although they retained their membrane shape and contained a soluble protein marker. We have thus established a direct role for AP3 in transporting membrane proteins to acidocalcisomes. This reinforces the similarity between acidocalcisomes and lysosome-related compartments, such as platelet dense granules, and raises the question of the origin of these various organelles.

Results

Generation of an AP3δ-null mutant cell lines and re-expression of AP3δ

The genome of *L. major* contains genes coding for sub-units of the AP1, AP2 and AP3 complexes (Ivens et al., 2005) (supplementary material Table S1) but apparently not of AP4. Disruption of any one of the AP3-complex genes in *Saccharomyces cerevisiae*, *D. melanogaster*, and also in mice and humans, led to misrouting of lysosomal membrane proteins (Boehm and Bonifacino, 2002; Cowles et al., 1997; Darsow et al., 1998; Stepp et al., 1997), indicating that each of the four AP3 subunits is equally important for the function of the complex. Thus, we aimed to disrupt AP3-complex function in *L. major* by deletion of the single gene coding for its δ subunit (*LmjF08.0090*). *L. major* is diploid and *AP3δ*-null mutants were generated after two rounds of homologous recombination, to replace each allele, using independent antibiotic selectable markers (Fig. 1A). Antibiotic-resistant parasites obtained after each round of transfection were cloned and analysed by Southern blotting (Fig. 1A) and by PCR (data not shown) to check for correct integration of the knockout cassettes. *Nae*I-digested genomic DNA from *L. major*, a heterozygote (Δ*ap3δ*/*AP3δ*) and a null-mutant clone (Δ*ap3δ*) were hybridised with a probe specific to the 5′ end of the *AP3δ* gene (Fig. 1A). The 4.7 kb DNA fragment corresponding to the *AP3δ* gene was detected in *L. major* and Δ*ap3δ*/*AP3δ* but not Δ*ap3δ*. The membrane was stripped and re-hybridised with a probe specific for the hygromycin-resistance gene, which also revealed an expected 4.85 kb DNA fragment for the Δ*ap3δ*/*AP3δ* and Δ*ap3δ* mutants (Fig. 1A). This indicates the efficient removal of the *AP3δ* locus in the Δ*ap3δ* cell line. This was further confirmed by assessing the expression of the gene by reverse transcriptase (RT)-PCR. The *AP3δ* gene could be amplified from cDNA from wild-type and Δ*ap3δ*/*AP3δ* parasites with primers specific for *AP3δ*, but not from cDNA from Δ*ap3δ*, showing that the gene was not expressed in the mutant (Fig. 1B). An *L. major* cell line was generated in which the *AP3δ* gene was re-expressed from the ribosomal locus in the Δ*ap3δ* parasites (designated Δ*ap3δ*::P_{RRNA} *AP3δ*). Expression of *AP3δ* in Δ*ap3δ*::P_{RRNA} *AP3δ* was confirmed by RT-PCR (Fig. 1B).

The MVT-lysosome seems unaffected in the AP3δ-deficient cell line

The AP3 complex has been found in several eukaryotes to be involved in sorting events towards the lysosome or lysosome-related organelles (Boehm and Bonifacino, 2002). We thus assessed the integrity of the lysosomal compartment in the Δ*ap3δ* promastigotes.

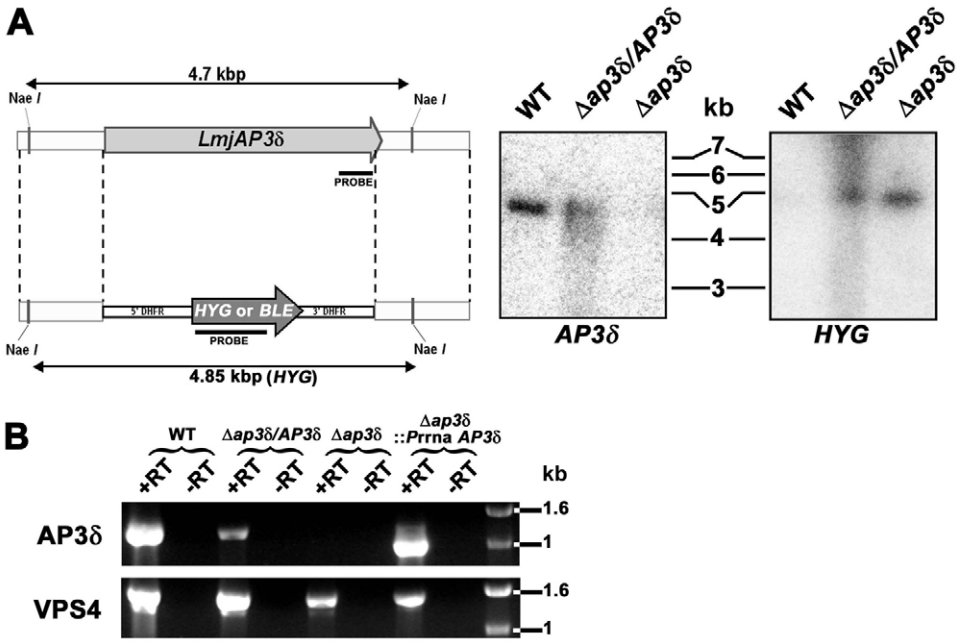

Fig. 1. Targeted replacement of the *AP3δ* gene. (A, from left to right) Schematic representation of the *AP3δ* locus and the constructs used for gene replacement. Genes are shown as arrows, intergenic and flanking DNA sequences as boxes. The restriction sites and the locations of the probes used for the Southern blot analyses, as well as the expected sizes of the labelled fragments, are displayed. *HYG*, hygromycin-resistance gene; *BLE*, phleomycin-resistance gene; *DHFR*, dihydrofolate reductase gene. Southern blot analyses of *L. major* wild-type (WT), *AP3δ* heterozygote (Δ*ap3δ*/*AP3δ*) and *AP3δ*-null mutant (Δ*ap3δ*) with probes specific for *HYG* and *AP3δ*. (B) RT-PCR analysis of specific mRNA expression in promastigotes of the cell lines described in A and the additional *AP3δ* re-expressing cell line (Δ*ap3δ*::P_{RRNA} *AP3δ*). Re-expression of *AP3δ* is from the ribosomal DNA locus and contains a different 3′UTR from the wild type gene, so the *AP3δ* mRNA is a different size. +RT and –RT, initial reaction with and without reverse transcriptase, respectively. *VPS4*-specific primers were used as a positive control.

The lysosome of the promastigote form of *Leishmania* contains several proteolytic enzymes, such as the cathepsin L-like peptidase CPB (Mullin et al., 2001). The main cellular peptidases activities were detected by gelatin-gel zymography and digestion profiles (Fig. 2A). Metallopeptidase (Fig. 2A, asterisk) and mature cysteine peptidase CPB (Fig. 2A, arrowhead) activities were found to be similar between wild-type and Δ*ap3δ* cell lines. Maturation of *L. major* CPB was further assessed by performing ^{35}S cellular metabolic labelling followed by various incubation times and, finally, by immunoprecipitation with an anti-CPB antibody (Mottram et al., 1996). CPB maturation was identical in wild-type and Δ*ap3δ* cell lines, with precursor CPB (Fig. 2B, asterisks) diminishing with time and mature CPB (Fig. 2B, arrowheads) appearing progressively at ~39 kDa as expected (Rafati et al., 2001). Overall these data show that maturation and activation of lysosomal CPB cysteine peptidase are not impaired in the Δ*ap3δ* mutant.

The lysosomal compartment of *L. major* promastigotes is characterised by a tubular shape when the parasites are in the stationary phases of growth (Ghedin et al., 2001; Mullin et al., 2001). Stationary-phase Δ*ap3δ* promastigotes had the typical tubular structure, when labelled with the lipophilic dye FM4-64 (Fig. 2C). In *S. cerevisiae*, the AP3 complex has been shown to be involved in the trafficking of membrane-bound vacuolar syntaxin Vam3p (Darsow et al., 1998). We recently identified in *L. major* two syntaxins that, once fused with the green fluorescent protein (GFP), localised to the MVT-lysosome (Besteiro et al., 2006a). Once expressed in the Δ*ap3δ* cell line, these two GFP-fused syntaxins were found to display a tubular signal identical to the localisation found in wild-type cells (Fig. 2C) (Besteiro et al., 2006a), showing that they were properly trafficked to the MVT-lysosome. This confirms that the MVT-lysosome retains its tubular structure in the Δ*ap3δ* mutant. It also suggests that the trafficking of lysosomal syntaxins appears to be independent of the AP3 machinery in *Leishmania*; however, a rerouted traffic of these syntaxins through the plasma membrane, as described for AP3-transported proteins LAMP I and LIMP II in mammalian cells (Le Borgne et al., 1998), could also explain the findings and deserves consideration.

Δ*ap3δ* promastigotes have a defect in their acidic potential

In eukaryotes, the AP3 complex is involved in the transport of several integral membrane proteins. As these potentially include transporters or ion channels, which in turn could be involved in the regulation of cellular pH, we aimed to assess the overall acidity of Δ*ap3δ* promastigotes. In *Leishmania* promastigotes the MVT-lysosome is thought to have a relatively high lumenal pH and Lysotracker was previously found to be mostly labelling acidocalcisomes, rather than the lysosome (Mullin et al., 2001). Indeed, Lysotracker labelled acidocalcisome-like vesicles in wild-type and re-expressing cell lines, but not Δ*ap3δ* (Fig. 3A). In some cases Lysotracker labelled the MVT-lysosome in Δ*ap3δ* parasites (Fig. 3A). In the control cell lines, this signal was generally masked by the stronger acidocalcisomal signal, which suggested that Δ*ap3δ* promastigotes had a higher acidocalcisomal pH than the wild type and AP3δ–re-expressing cell lines. This result was confirmed by labelling with Acridine Orange, a tertiary amine that becomes concentrated in acidic compartments and has been shown to label acidocalcisomes in trypanosomatids (Docampo et al., 1995). Δ*ap3δ* promastigotes showed a greatly reduced number of labelled puncta and a less intense signal with Acridine Orange compared with wild-type and AP3δ–re-expressing cell lines (Fig. 3A).

To further assess the acidic potential of the different cell lines we used Neutral Red, a dye previously used to measure intracellular acidity in living cells (Burkhardt et al., 1993; Lemercier et al., 2002). In acidic environments (i.e. pH 5), the Neutral Red absorbance peak shifts to 540 nm compared with 450 nm at basic pH (i.e. pH 9). A Neutral Red absorbance of almost half was observed at 540 nm in the Δ*ap3δ* cell line compared with that in wild type and the re-expresser cell lines (Fig. 3B), indicating a substantial reduction of acidic capacity in the mutant cells.

Acidocalcisomes are an important storage compartment for polyP and it has been hypothesised that they have a role in the adaptation of the parasites to environmental stress (Docampo et al., 2005; Ruiz et al., 2001). When the pH of acidocalcisomes is alkalinised, there is a concomitant disappearance of polyP content (Lemercier et al., 2002; Ruiz et al., 2001). Indeed, polyP content

was found to be much lower in Δ*ap3δ* cells compared with wild-type or re-expresser cell lines (Fig. 3C).

Δ*ap3δ* promastigotes lack acidocalcisomal membrane proteins

The weak labelling with the acidophilic dyes in the Δ*ap3δ* cells suggested that either the internal pH of the acidocalcisomes was higher or that the mutant cell lines lacked these organelles. To assess this, the presence of acidocalcisomal markers, including the two membrane proteins vacuolar proton pyrophosphatase (V-H$^+$-PPase) (Lemercier et al., 2002) and vacuolar proton ATPase (V-H$^+$-ATPase) (Docampo et al., 1995; Vercesi et al., 1994), was assessed. The V-H$^+$-PPase is an electrogenic proton pump first discovered in the vacuole of plants, but it has also been found in photosynthetic bacteria and in the membrane of acidocalcisomes of trypanosomatids (Rodrigues et al., 1999a; Rodrigues et al., 1999b; Scott et al., 1998). Using an antibody raised against *T. brucei* V-H$^+$-PPase (Lemercier et al., 2002), two protein bands were detected in *L. major* wild-type promastigotes extracts, a major signal at ~70 kDa and a minor one at ~55 kDa (Fig. 4A). The lower molecular-mass band might stem from an isoform of the V-H$^+$-PPase (Lemercier et al., 2002) or possibly an unrelated cross-reacting protein. In cell extracts from the Δ*ap3δ* cell line, the ~70 kDa V-H$^+$-PPase was significantly reduced (Fig. 4A), whereas the ~55 kDa protein was retained (Fig. 4A). Cell fractionation and differential-centrifugation experiments showed that the ~70 kDa isoform was associated with the acidocalcisome fraction, whereas the ~55-kDa protein was not (supplementary material Fig. S1). Indeed, when used for immunolocalisation, the V-H$^+$-PPase antibody detected fewer acidocalcisomes at lower intensity in Δ*ap3δ* compared with the wild-type and *AP3δ*-re-expressing promastigotes (Fig. 4B). It is possible that the signal detected in Δ*ap3δ* is due to the presence of the ~55-kDa protein.

To confirm the inability of V-H$^+$-PPase to be trafficked to the acidocalcisomes in the Δ*ap3δ* cells, we expressed a GFP fusion of this protein in *L. major*. The V-H$^+$-PPase-GFP fusion protein localised to acidocalcisomes in wild-type parasites (shown by colocalisation with Lysotracker), whereas no significant labelling was detected in Δ*ap3δ* cells (Fig. 4C). Expression of V-H$^+$-PPase-GFP in the cell lines was confirmed by GFP-specific RT-PCR (data not shown). The localisation of V-H$^+$-PPase-GFP is thus consistent with that obtained for the wild-type V-H$^+$-PPase using a specific antibody (Fig. 4B).

AP complexes recognise their cargo proteins through specific motifs such as di-leucine-based or tyrosine-based (YxxΦ, where Φ is an amino acid with a bulky hydrophobic side chain) sorting signals (Bonifacino and Traub, 2003). When we investigated the *L. major* V-H$^+$-PPase we could find four putative tyrosine-based motifs (^{115}YTRI^{118}, ^{130}YKYM^{133}, ^{467}YRPV^{470}, ^{539}YGPI^{542}), some of which are also conserved in other trypanosomatid species and can potentially serve as AP-recognition motifs. This thus suggests that AP3 is involved in V-H$^+$-PPase trafficking, although this needs further detailed study.

The pyrophosphate (PPi)-dependent H$^+$-pumping activity of the V-H$^+$-PPase can be assessed on permeabilised cells using Acridine Orange to spectrophotometrically detect the acidification of the acidocalcisomes (Lemercier et al., 2002; Rodrigues et al., 1999a). The decrease in absorbance observed for the wild-type and Δ*ap3δ*::P_{RRNA} *AP3δ* permeabilised promastigotes upon addition of PPi could be reversed by adding alkalinising agent NH_4Cl (Fig. 5A) or the ionophore nigericin (data not shown), showing that it was indeed due to an H$^+$ gradient. However, in the Δ*ap3δ* promastigotes no PPi-dependent H$^+$ gradient could be observed (Fig. 5A).

The activity of the V-H$^+$-ATPase (Docampo et al., 1995; Vercesi et al., 1994) was also assessed. ATP-driven H$^+$ accumulation was visualised spectrophotometrically through change in the absorbance of Acridine Orange in wild-type and *AP3δ*-re-expressing permeabilised promastigotes and the gradient was reverted by alkalinisation with

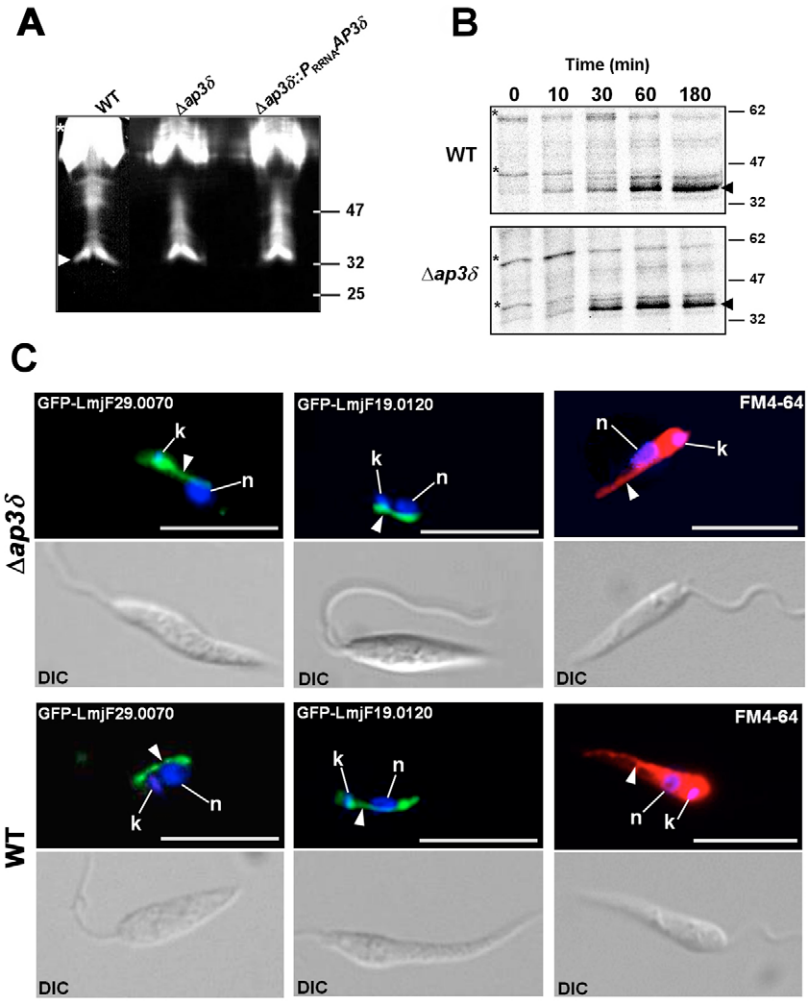

Fig. 2. Normal lysosome function in the Δ*ap3δ* mutant. (A) Gelatin-SDS-PAGE analysis to detect the activities of mature lysosomal cysteine peptidase CPB (arrowhead) and metallopeptidases (asterisk). (B) Metabolic labelling and immunoprecipitation analysis assessing the maturation of CPB in the wild-type and Δ*ap3δ* cell lines. The mature CPB form is indicated by an arrowhead and the precursor forms by asterisks. Molecular masses are indicated on the right. (C) Labelling of the MVT-lysosome (arrowheads) in *L. major* Δ*ap3δ* and wild-type promastigotes with GFP-tagged syntaxins LmjF19.0120 and LmjF29.0070 (green signal) and endocytic tracer FM4-64 (red signal). n, nucleus; k, kinetoplast; scale bars, 10 μm. Corresponding differential interference contrast (DIC) images are shown as well.

NH_4Cl (Fig. 5B). However, Δ*ap3δ* promastigotes displayed a much reduced activity (Fig. 5B), showing that the V-H^+-ATPase was affected in the mutant.

The soluble acidocalcisomal protein VSP1 is found in acidocalcisomes of Δap3δ promastigotes

VSP1 is a soluble pyrophosphatase found in *T. brucei* (Lemercier et al., 2004) and *L. amazonensis* (Espiau et al., 2006) acidocalcisomes. The ~50-kDa VSP1 was detected in *L. major* wild-type, *AP3δ*-null mutant and *AP3δ*–re-expressing cell extracts with an anti-VSP1 antibody (Fig. 6A). The antibody also detected acidocalcisomes in the three cell lines (Fig. 6B), and both fluorescence intensity and number of organelles were generally found to be similar between the three cell lines. This demonstrates that the targeting of VSP1 to acidocalcisomes is unaffected in Δ*ap3δ* promastigotes, and that acidocalcisomes appear to be present in the mutant. Electron-spectroscopic imaging of live promastigotes (Fig. 6C) showed that Δ*ap3δ* cells lacked the electron-dense material in their acidocalcisomes that is typically observed in wild-type cells. This is consistent with the absence of polyphosphate from Δ*ap3δ* cells, which has been shown previously to be a major component of the electron-dense material (Fig. 3C). Standard transmission electron microscopy confirmed that membrane-bound acidocalcisomes themselves were still present in the mutant, displaying vacuoles of similar size; but clearly their content was very different from the organelles in wild-type and AP3δ-re-expressing cells because they were devoid of dense material (Fig. 6C).

The AP3 complex is not essential for in vitro viability, differentiation and macrophage invasion but for disease progression in mice

The Δ*ap3δ* promastigotes could be maintained in culture, showing that the AP3 complex is not essential for viability in vitro; however, they had a reduced growth rate. This growth phenotype could be complemented in the re-expressing cell line (Fig. 7A). At the stationary phase of growth, procyclic promastigotes differentiate into the infective, non-dividing metacyclic promastigotes. Δ*ap3δ* parasites expressed the metacyclic promastigote-specific protein HASPB (Flinn et al., 1994) (Fig. 7B) and displayed a typical metacyclic morphology (i.e. long flagellum and thin cell body; data not shown), indicating that the parasites can differentiate to this infective life cycle stage.

Late-stationary-phase promastigotes of the different cell lines were assessed in vitro for their ability to infect and survive in peritoneal macrophages explanted from CD1 mice. There was no significant difference in the mean number of macrophages infected with wild-type, Δ*ap3δ* or re-expressing cell lines after 1, 3 or 5 days, showing that the *AP3δ* gene is not essential for macrophage invasion and the subsequent survival of the parasites (Fig. 7C and data not shown). However, the average number of parasites per macrophage was significantly less with Δ*ap3δ* parasites than with wild type parasites after 5 days. The Δ*ap3δ* parasite numbers increased from 1.52±0.32 after 4 hours to 4.9±0.45 after 5 days, which was less than with the wild-type parasites (Fig. 7C). This could indicate a reduced ability of Δ*ap3δ* cells to multiply within the macrophages compared with the wild-type and re-expressing cell lines (Fig. 7C). Overall, these data indicate that the Δ*ap3δ*-mutant promastigotes can transform into infectious metacyclic forms in vitro and differentiate into the amastigote forms in macrophages; they are, however, less able to replicate intracellularly.

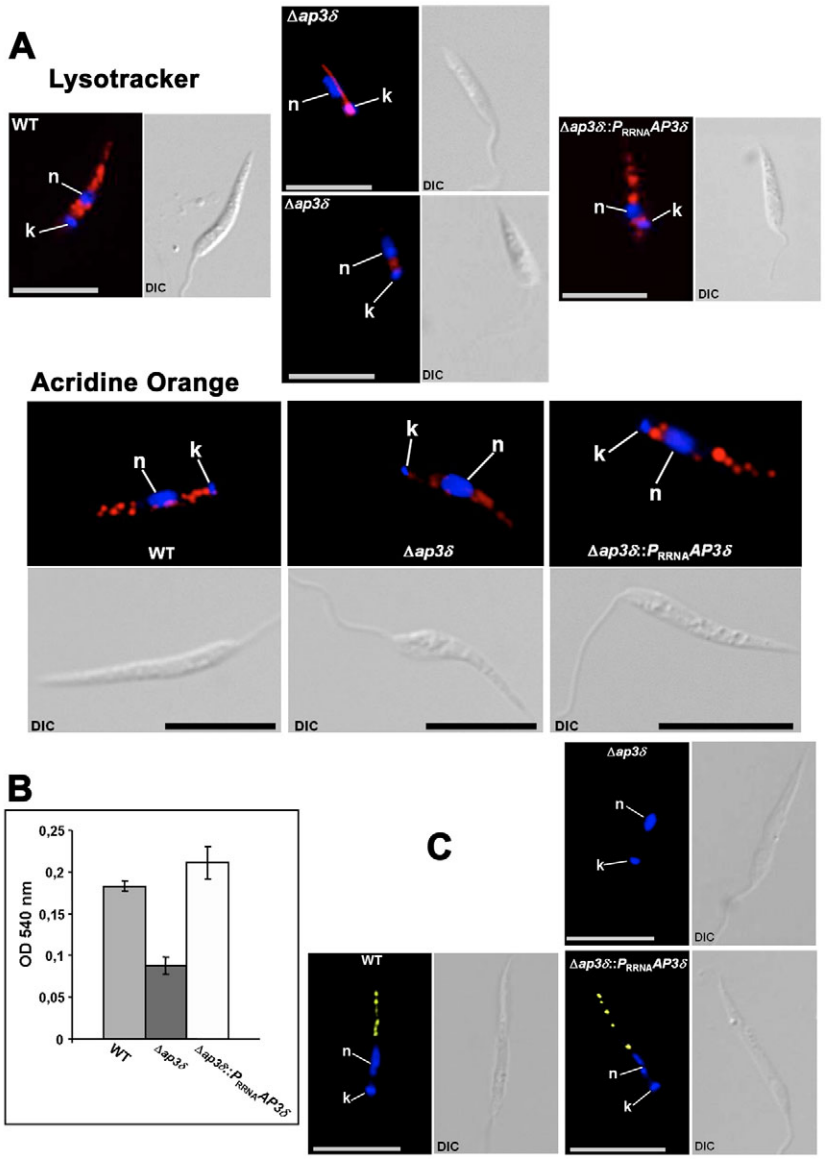

Fig. 3. Acidocalcisome defects in Δ*ap3δ* promastigotes. (A, top panels) Lysotracker labelling of acidic compartments in *L. major* promastigotes. The two types of signals observed for the Δ*ap3δ* cell line, including the MVT-lysosome-like tubular labelling, are displayed in the four middle panels. (A, bottom panels) Acridine-Orange-labelling of acidic compartments in *L. major* promastigotes. (B) Measurements of Neutral Red change in absorbance at 540 nm due to acidic environments of *L. major* promastigotes. Data represent the means ± standard deviations (s.d.) from four independent experiments. (C) Visualisation of polyP-enriched vesicles (yellow) with DAPI in *L. major* promastigotes. n, nucleus; k, kinetoplast. All scale bars, 10 μm. Corresponding DIC images are shown as well.

The ability of the transgenic parasites to cause lesions in mice was also investigated. Equal numbers of stationary-phase parasites (5×10^5) were injected into the footpad of susceptible mice and lesion development was monitored (Fig. 7D). The Δ*ap3δ* parasites formed only small lesions over an 8-week period, whereas wild-type and Δ*ap3δ*::P_{RRNA}*AP3δ* parasites formed large lesions within 4 weeks. Footpads were dissected and parasite numbers were found to be consistent with footpad size (wild-type parasites at week 4, $2.45\times10^8\pm6.7\times10^7$; Δ*ap3δ* parasites at week 8, $2.46\times10^7\pm1.98\times10^6$; Δ*ap3δ*::$P_{RRNA}$*AP3δ* at week 4, $2.3\times10^8\pm4.06\times10^7$ parasites per lesion). Thus, although Δ*ap3δ* parasites could establish an infection in the mice they were only able to multiply slowly.

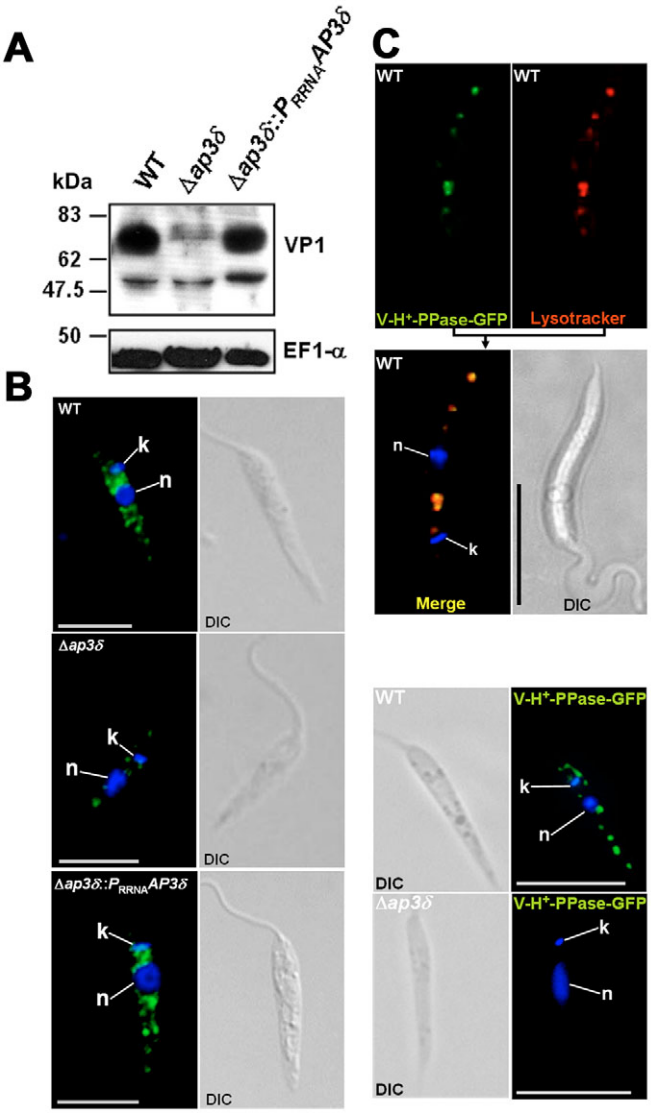

Fig. 4. Acidocalcisomal membrane V-H$^+$-PPase is downregulated in Δ*ap3δ* promastigotes. (A) Western blot analysis of whole-cell lysates from *L. major* promastigotes with the anti-V-H$^+$-PPase antibody (VP1). Elongation factor 1α (EF1-α) was used as a loading control. Molecular sizes are indicated on the left. (B) Immunofluorescence analysis of *L. major* promastigotes using the anti-V-H$^+$-PPase antibody. (C) Localisation in WT and Δ*ap3δ L. major* promastigotes, of the V-H$^+$-PPase with GFP fused at its C-terminal end. All scale bars, 10 μm; n, nucleus; k, kinetoplast. Corresponding DIC images are shown as well.

Discussion

AP3 is a heterotetrameric protein complex that recognises sorting signals for trafficking of several integral membrane proteins to the yeast vacuole, insect pigment granules and – in mammals – to lysosomes, melanosomes, dense granules and other lysosome-related organelles as well as synaptic vesicles of neurons. However, the targeting of two syntaxins that are lysosomal membrane proteins (Fig. 2C), the processing and activity of the lysosomal soluble peptidase CPB (Fig. 2A,B), and the overall structure of the lysosomal compartment analysed using FM4-64 (Fig. 2C) all appeared normal in Δ*ap3δ Leishmania* cells in which both alleles encoding the δ subunit of the AP3 complex had been deleted by genetic manipulation. These findings are suggestive of a functional lysosomal compartment in the *Leishmania* mutant, and this is consistent with other systems, such as yeast and mammalian cells, where the loss of AP3 does not significantly affect lysosome morphology or content (Cowles et al., 1997; Le Borgne et al., 1998). However, we cannot at this stage rule out the possibility that the targeting of some lysosomal membrane proteins is affected in the Δ*ap3δ Leishmania* parasites. This is difficult to assess currently because only a few lysosomal AP3-transported proteins have been identified for any eukaryote, and few of these appear to have leishmanial homologues. Further studies will be required to evaluate the extent to which the lysosomal compartment of the Δ*ap3δ* cells is affected and to investigate the role of AP3 in the biogenesis of the lysosomal compartment in the parasite.

Clearly, however, the Δ*ap3δ* cells have defective acidocalcisomes, acidic organelles that contain high concentrations of phosphorus (in the form of PPi and polyP) complexed with Ca^{2+}

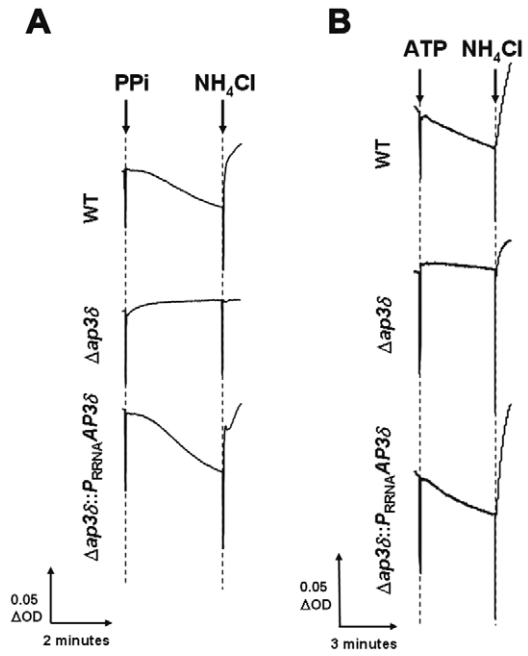

Fig. 5. Δ*ap3δ* promastigotes are defective in PPi- and ATP-dependent proton transport. (A,B) Proton-pumping activities visualised in permeabilised promastigotes loaded with Acridine Orange, upon addition of (A) 500 μM $NaPP_i$ or (B) 1 mM ATP. 20 mM of NH_4Cl was added to reverse the proton gradient when indicated. The horizontal scales represent time and the vertical scales the absorbance variations of Acridine Orange at 530 nm and 493 nm following pH changes. Data shown are representative of three experiments performed with independent cultures.

and other cationic elements. Their composition is complex, varies between species and growth conditions (Miranda et al., 2004), and their functions are not yet precisely elucidated. Among the proposed roles for acidocalcisomes are (1) storage of phosphorus, various ions, metals and amino acids; (2) osmoregulation and, (3) maintenance of the intracellular pH. Although a number of membrane transporters have been analysed, the protein content of acidocalcisomes is still poorly characterised and nothing was known about the targeting of proteins to the organelle. We have now shown that the AP3 complex is involved in transporting acidocalcisomal membrane proteins. Both V-H^+-ATPase and a V-H^+-PPase, usually acidocalcisome membrane proteins, have a much lower activity in the Δ*ap3δ* cells. Indeed, the latter protein could not be detected at all in the acidocalcisomes of Δ*ap3δ* cells. Moreover, both the V-H^+-PPase and V-H^+-PPase-GFP were detected only at very low levels in Δ*ap3δ* cells compared with those in wild-type cells; perhaps because the proteins were not targeted properly and rapidly degraded in the promastigotes. The peptidases responsible for this degradation are under investigation. Of note is the recent finding that several multispanning transmembrane proteins, including V-H^+-PPase, were mislocalised in a *T. brucei* signal-recognition particle (SRP)-deficient mutant (Lustig et al., 2007). The authors have shown that in the SRP-deficient mutant the V-H^+-PPase was not trafficked to acidocalcisomes but possibly degraded by an unidentified peptidase.

Another consequence of the disruption of AP3 function in *Leishmania* promastigotes was the disappearance of the polyP (as seen by DAPI-labelling, Fig. 3C) and electron-dense content of the acidocalcisomes (as seen by electron microscopy; Fig. 6C). This might be linked to the defects in the two H^+-pumping activities we have assessed [V-H^+-PPase RNAi mutants also had diminished acidocalcisomal polyP content (Lemercier et al., 2002)] or might be owing to an alteration in the function of other acidocalcisomal membrane proteins/transporters such as membrane vacuolar transporter chaperone 1, recently characterised in *T. brucei* (Fang et al., 2007), or others yet-to-be characterised in *Leishmania*.

The Δ*ap3δ* promastigotes grew well in vitro, showing that the AP3 complex is not essential for this. However, whereas the parasites could also differentiate into infective metacyclic forms and infect macrophages well in vitro (albeit the amastigotes of the mutant line growing more slowly than wild-type parasites), they grew considerably slower in mice. It is tempting to suggest that the attenuated virulence of Δ*ap3δ* amastigotes reflects the defect in their acidocalcisomes and, indeed, we have been able to detect by DAPI staining a loss of polyphosphate in Δ*ap3δ* amastigotes residing in macrophages in vitro (data not shown). There are other reports on a role for acidocalcisomes in the virulence of *Leishmania*. For example, *Leishmania* mutants of polyphosphate-regulating soluble pyrophosphatase (Espiau et al., 2006), although readily invading macrophages in vitro, were unable to produce lesions in mice. One can speculate that acidocalcisomes play a role in countering environmental changes, such as osmotic stress, that certainly occur during the establishment of an infection.

Crucial functions of acidocalcisomes are altered in Δ*ap3δ* cells, but the use of soluble acidocalcisomal pyrophosphatase as a marker and ultrastructural observations indicate that acidocalcisomes are still present in the Δ*ap3δ* promastigotes (Fig. 6). This suggests that the AP3 complex, although involved in transporting

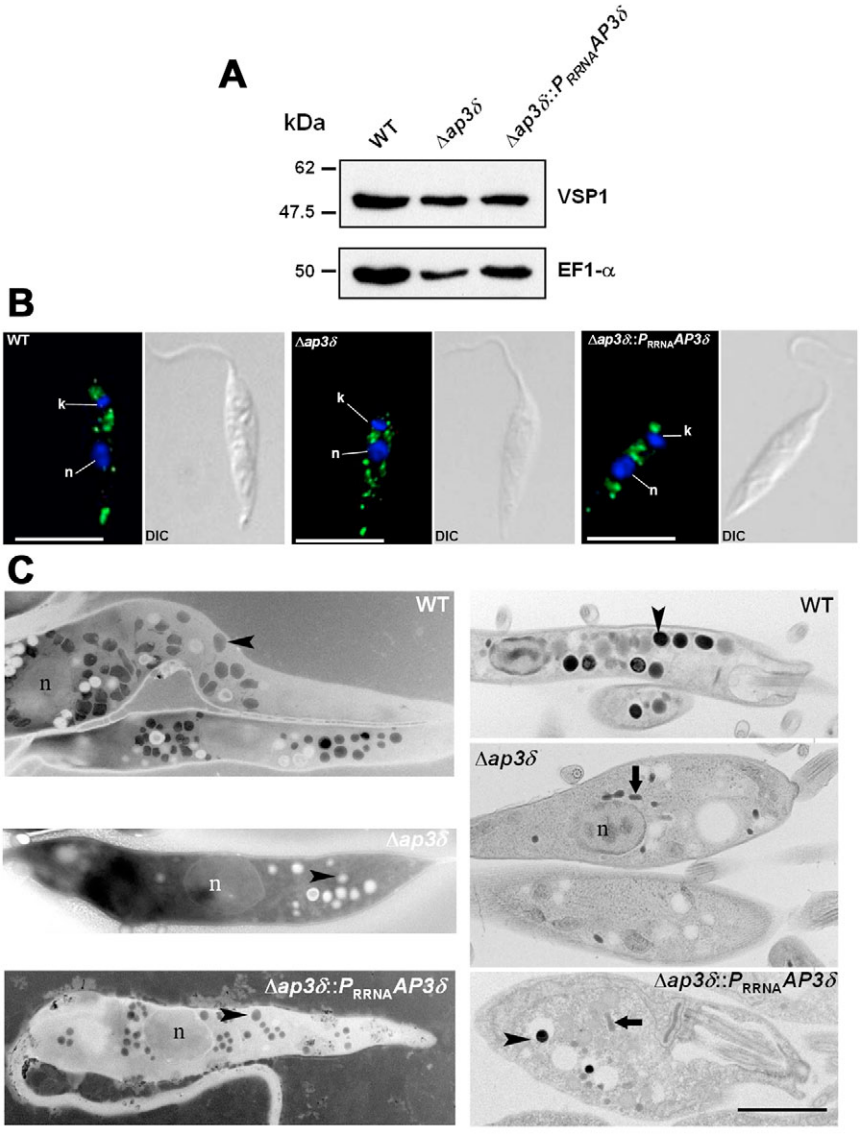

Fig. 6. Acidocalcisomes from Δ*ap3δ* promastigotes contain a soluble pyrophosphatase, but are devoid of electron-dense material. (A) Western blot analysis of whole-cell lysates from *L. major* promastigotes with the anti-VSP1 antibody. EF1-α was used as a loading control. Molecular masses are indicated on the left. (B) Immunofluorescence analysis of *L. major* promastigotes using anti-VSP1 antibody. The corresponding DIC images are shown. (C, left panels) electron spectroscopic whole cell imaging of WT, Δ*ap3δ* and Δ*ap3δ*::P_{RRNA} *AP3δ* promastigotes showing presence or absence of electron-dense polyphosphate in acidocalcisomes (arrowheads). (Right panels) Corresponding transmission electron microscopy of sections of these cell types. Acidocalcisomes are indicated with arrowheads. Nuclei (n) and glycosomes (arrows) are indicated as other organelles for reference; k, kinetoplast. Scale bars, 10 μm (B) and 2.0 μm (C).

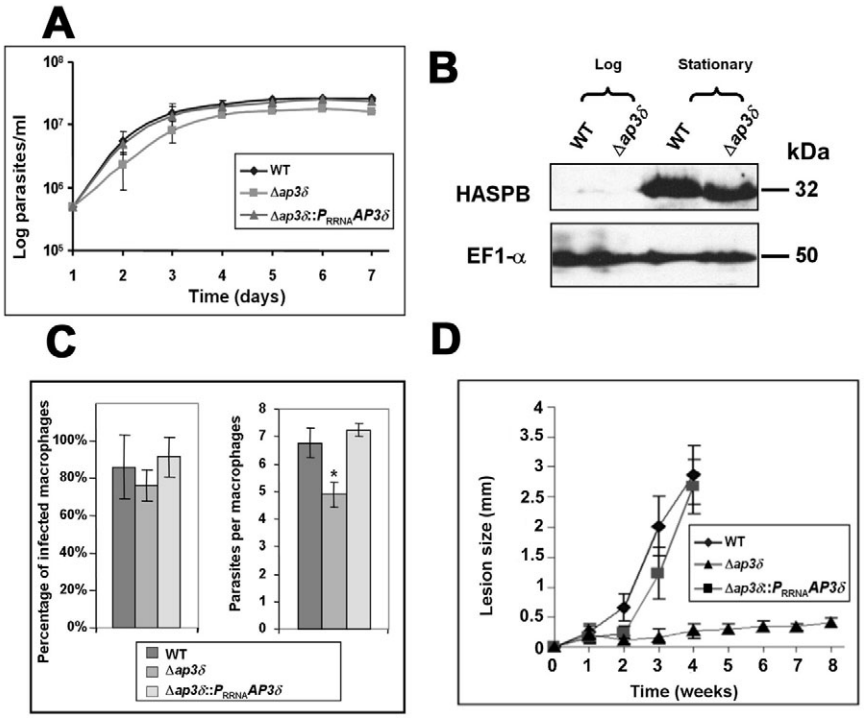

Fig. 7. AP3δ is a virulence factor. (A) In vitro growth of promastigotes. Data shown represent the means ± s.d. from three independent series of measurements. (B) Western blot analysis with metacyclic-specific anti-HASPB antibody. EF1-α was used as a loading control. Molecular masses are indicated on the right. (C) In-vitro-macrophage-infectivity assay. Stationary-phase parasites were used to infect explanted mouse peritoneal macrophages at a 3:1 ratio for 4 hours. The proportion of infected macrophages (left) and the number of parasites per infected macrophage (right) were determined after 5 days of incubation. Data shown represent are the means ± s.d. from triplicate infections. *, results for Δ*ap3δ* cells that differ significantly from those for the other two cell lines (*t*-test, $P<0.05$). (D) Infection of CD1 mice with wild-type and transgenic stationary-phase *L. major* promastigotes. Mice were challenged with 5×10^5 parasites in the left hind footpad. The swelling caused by the respective cell lines was recorded. Data shown represent mean lesion depth ± s.d. from groups of six mice.

crucial acidocalcisomal membrane proteins, is not essential for the biogenesis of the organelle itself. The origin of the acidocalcisomes is still debated. Endocytic tracers such as FM4-64 appear not to accumulate in these organelles in wild-type *Leishmania* (Mullin et al., 2001). However, acidocalcisomes from sterol biosynthesis inhibitor-treated parasites were shown to accumulate endocytic markers (Vannier-Santos et al., 1999), which suggests there is some association of acidocalcisomes with the endosomal/lysosomal pathway. A *L. major* sphingolipid-deficient mutant has been shown to be defective in the biogenesis of both multivesicular bodies (or late endosomes) and acidocalcisomes (Zhang et al., 2005), which suggests that these compartments have a common origin.

Indeed, by showing that the system known to be involved in transport of membrane proteins to lysosomes and lysosomes-related organelles in other cells has a similar function with respect to acidocalcisomes in *Leishmania*, our results provide support for a close similarity between acidocalcisomes and the endo/lysosomal system. This potential link warrants further investigation. In fact, in many of their properties, acidocalcisomes strikingly resemble lysosome-related organelles. It has been judiciously pointed out by others that platelet dense granules – lysosome-related secretory compartments involved in blood coagulation – are very similar to acidocalcisomes (Ruiz et al., 2004; Zhang et al., 2005). Notably, they share a similar size, acidic properties, and both contain polyP and Ca^{2+}. Our findings that they also share the system for targeting of their membrane proteins reinforce this concept. Further evidence is provided by the finding that the Hermansky-Pudlak syndrome, a human autosomal recessive disorder characterised by oculocutaneous albinism and platelet-storage-pool deficiency, is due to mutations in one of the AP3-subunit genes (Dell'Angelica et al., 1999; Kantheti et al., 1998); thus AP3 is known to be important for the biogenesis of functional dense granules in platelets. Of particular note, the polyP from platelets has recently been shown to be involved in regulating clot formation and it was suggested that defects in dense granules are due to a lack of polyP (Smith et al., 2006). Given the conserved role of AP3 in the function of both dense granules and acidocalcisomes, and the defect in polyP content of the acidocalcisomes we observed in our Δ*ap3δ* mutant, we propose that the prolonged bleeding associated with the Hermansky-Pudlak-like disorders in mammals is due to a lack of polyP in the dense granules of platelets.

These findings, which provide additional support for the relatedness of acidocalcisomes to other lysosome-related organelles, suggest that further insights into acidocalcisomes could also shed light on conserved functions in similar organelles of other cells of medical relevance. In turn, an analysis of some of the features of various lysosome-related organelles, especially their secretory properties, are likely to highlight as-yet-undiscovered functions of acidocalcisomes that will aid in the understanding of how acidocalcisomes play a part in determining the virulence of protozoan parasites.

Materials and Methods

Parasites

L. major (MHOM/JL/80/Friedlin) promastigotes were grown in modified Eagle's medium (MEM) with 10% (v/v) heat-inactivated foetal calf serum (FCS) – designated complete HOMEM – at 25°C. Macrophage infections were carried out as described previously (Besteiro et al., 2004).

Generation of *L. major* Δap3δ-null mutant and re-expressing cell lines

The 500 bp 5′ flank fragment of *LmjAP3δ* (*LmjF08.0090*) was generated by PCR from *L. major* DNA with primers OL1801 and OL1802 (all oligonucleotides used in this study can be found in supplementary material Table S2), digested with *Hin*dIII and *Sal*I and inserted into *Hin*dIII/*Sal*I-digested pGL345-HYG bearing the hygromycin-resistance gene (Mottram et al., 1996), to give pGL1282-5′. The 3′ fragment was generated by PCR using primers OL1803 and OL1804. The resulting 500 bp fragment was digested by *Sma*I and *Bgl*II and cloned into pGL1282-5′ to give pGL1282. The cassette used for transfection was released by *Hin*dIII/*Bgl*II digestion. The pGL1283 plasmid, used for the replacement of the second *AP3δ* allele, was generated from plasmid pGL1282 by replacing the *Spe*I/*Bam*HI cassette

containing the hygromycin-resistance gene with a *Spe*I/*Bam*HI cassette containing the phleomycin-resistance gene.

To generate a re-expressing cell line, a copy of *AP3δ* was cloned into the pRIB expression vector (Garami and Ilg, 2001). *AP3δ* was obtained by PCR from *L. major* DNA using the Phusion DNA polymerase (Finnzymes) with primers OL2066 and OL2067 containing *Sal*I and *Bgl*II restriction sites, respectively. The 3.4 kb fragment obtained was sequenced and then digested with *Sal*I/*Bgl*II before cloning into *Xho*I/*Bgl*II-digested pRIB to yield plasmid pGL1490. The integration cassette from this plasmid was excised by *Pac*I/*Pme*I digestion, gel-purified and 40 μg were transfected into *L. major* Δ*ap3δ* promastigotes. *Leishmania* genetic manipulations were carried out as described previously (Hilley et al., 2000).

Generation of *L. major* cell lines expressing V-H$^+$-PPase-GFP

The *L. major V-H$^+$-PPase* gene (*LmjF31.1220*) was obtained by PCR from genomic DNA with primers OL2426 and OL2427 containing *Nde*I and *Kpn*I restriction sites, respectively. After digestion, the PCR fragment was cloned into *Nde*I/*Kpn*I-digested pNUS-GFPcN vector (Tetaud et al., 2002) allowing C-terminal fusion of GFP, resulting in the new plasmid pGL1681. *L. major* wild type and Δ*ap3δ* mutant cells were transfected with the plasmids as described previously (Besteiro et al., 2006b).

Southern blot analysis of transfectants

DNA was isolated from the transfectants with the DNeasy kit (Qiagen). Three micrograms were digested with *Nae*I, electrophoresed through a 0.8% agarose gel and blotted onto Hybond-N+ membrane (Amersham Pharmacia). A 472 bp *AP3δ*-specific probe corresponding to the 3′ end of the gene was generated from a PCR fragment obtained with primers OL1872 and OL2067. The fragment was labelled with a random priming kit (Stratagene) and the blot was hybridised at 65°C overnight. The *HYG* gene-specific probe was produced as described previously (Besteiro et al., 2004). The membrane was washed for 15 minutes at 65°C with 2×SSC with 0.1% SDS and then twice with 0.2×SSC with 0.1% SDS.

RNA extraction and RT-PCR

Total RNA was extracted from ~10^8 promastigotes using the TRIzol reagent (Invitrogen). Samples were then treated with RQ1 RNase-free DNase (Promega) to remove any contaminating DNA. After a phenol-chloroform extraction, RNA was precipitated and 5 μg were used to synthesise first strand of cDNA with SuperScript III reverse transcriptase (Invitrogen). One-tenth of the final material was used as a template for separate PCR reactions using the splice-leader primer (OL1760) and a reverse primer specific for *AP3δ* (OL2069) or the positive control *LmjVPS4* (Besteiro et al., 2006b) (OL1479). Of each reaction 1% was used as a template for a round of semi nested-PCR amplification using an internal primer: splice-leader oligonucleotide (OL1760) was still used as the 5′ primer, together with a specific internal 3′ reverse primer for *AP3δ* (OL1967) or *LmjVPS4* (OL2148). Products of both series of reactions were resolved on a 1% agarose gel.

Metabolic labelling and immunoprecipitation

For metabolic labelling and immunoprecipitation of CPB, cells were treated as described previously (Ellis et al., 2002). 6×10^7 cells were resuspended in 1 ml of labelling medium [1× minimum essential medium (ICN), 2 mM L-glutamine, 10% (v/v) FCS (dialysed against PBS and 0.22 μm-filtered)], incubated for 20 minutes with 100 μCi of Expre^{35}S^{35}S ([^{35}S]methionine-[^{35}S]cysteine protein-labelling mix; NEN), and resuspended in an equivalent volume of MEM containing 10% (v/v) FCS at 25°C. 100 μl aliquots of cells were collected at appropriate time points and cells were stored at –20°C. For immunoprecipitation, the cell pellets were resuspended in 1× solubilisation buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitor cocktail (Roche)] to 1 ml, incubated 10 minutes on ice and centrifuged at 10,000 ***g*** for 15 minutes to pre-clear. 50 μl of protein A/G-Sepharose (resuspended to a concentration of 0.5 mg/ml in solubilisation buffer) and 20 μg of anti-CPB antiserum of *L. mexicana* (Mottram et al., 1996) were added to the supernatants and the samples were mixed at 4°C for 10 hours. They were then washed three times in TEN-D buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS) and once in TEN buffer (TEN-D buffer without detergent). Pellets were resuspended in 20 μl of SDS-PAGE loading buffer and analysed by gel electrophoresis.

Gelatin SDS-PAGE analysis

Cysteine peptidase activity was assayed by gelatin SDS-PAGE as previously described (Robertson and Coombs, 1990). Coomassie Blue was used to visualise the hydrolysis of 0.2% gelatin copolymerised in a 12% polyacrylamide separating gel.

Western blotting

Western blot analysis was performed on cell fractions or total cell extracts (from 10^7 promastigotes) with the anti-TbVP1 rabbit antibody (Lemercier et al., 2002) and rabbit anti-TbVSP1 (Espiau et al., 2006; Lemercier et al., 2004) diluted at 1:1000. Other western blot analyses involved the use of rabbit anti-HASP antibody (Flinn et al., 1994) at a 1:1000 dilution and mouse anti-TbEF1α antibody (Upstate) at 1:10,000.

Fluorescence staining of cells

For *N*-(3-triethylammoniumpropyl)-4-{6-[4-(diethylamino)phenyl]hexatrienyl} pyridinium dibromide (FM4-64) labelling, the cells were incubated with 40 μM of FM4-64 (from a 12 mM stock solution in DMSO; Invitrogen) for 15 minutes at 4°C and then washed in fresh medium and incubated for 30 minutes at 25°C. For Lysotracker labelling, cells were incubated for an hour at 25°C in HOMEM with 50 nM of Lysotracker (Invitrogen). For observation of Acridine-Orange-stained acidic compartments, cells were washed twice in a buffer containing 116 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM K-HEPES pH 7.4, and incubated for 15 minutes at 25°C in the same buffer supplemented with 6 μM Acridine Orange (Sigma). After incubation with the dyes or for observation of the GFP cell lines, cells were washed three times in cold phosphate buffered saline (PBS) before being processed for microscopy.

For DAPI labelling, fixed cells were incubated for 10 minutes with 10 μg ml^{-1} DAPI (Sigma) before being processed for microscopy. PolyP content of acidocalcisomes was detected specifically by confocal laser-scanning microscopy with an argon laser from a Zeiss LSM510 Meta laser system and with a Zeiss Axiovert 200M microscope. Images were then acquired with a Zeiss UV Axioskop 2 and a 100× objective using the DAPI filter (Tijssen et al., 1982).

For immunofluorescence analysis, cells were processed as described previously (Besteiro et al., 2004). Primary antibodies were incubated at the appropriate dilution, 1:1000 for rabbit anti-*Tb*V-H$^+$-PP (Lemercier et al., 2002) and 1:200 for rabbit anti-TbVSP1 (Espiau et al., 2006), in PBS with 0.1% BSA for 30 minutes, followed by three washes in PBS and subsequent incubation with fluorochrome-conjugated secondary antibodies. Cells were viewed with a Zeiss UV Axioskop 2 microscope and images were captured by an Orca-ER camera (Hamamatsu) and Openlab software v 5 (Improvision) and deconvolved using the volume deconvolution module.

Neutral-Red-uptake assay

10^7 promastigotes were incubated with 150 μM of Neutral Red (Sigma) in HOMEM for 30 minutes. After three washes in PBS, cells were lysed in 1 ml of water:ethanol:acetic acid (50:49:1) and the amount of dye incorporated was determined spectrophotometrically at 540 nm (Lemercier et al., 2002).

ATP- and PPi-dependent proton transport in permeabilised cells

Proton transport was assessed (Lemercier et al., 2002; Rodrigues et al., 1999a), by measuring the changes in absorbance of Acridine Orange using a HP8453 spectrophotometer at the wavelength pair 530-493, at 25°C. Acridine Orange was added at 1 μg ml^{-1} to 5×10^7 cells and the sample was then incubated with 50 mM digitonin or 20 μg ml^{-1} of filipin (Sigma) for two minutes. The reaction started with the addition of 1 mM ATP or 500 μM $NaPP_i$. NH_4Cl (20 mM) was added when indicated.

Electron microscopy

For whole parasite imaging, promastigotes were washed in PBS and resuspended in 250 mM trehalose. They were applied to a Formvar/carbon-coated grid and imaging was done at 120 kV in a Zeiss LEO 912 energy filtering transmission electron microscope at 30 eV. For sectioned parasites, promastigotes were fixed by adding a mixture of 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 40 minutes. Subsequent processing followed standard methods (Coombs et al., 1986). Resin sections (100 nm thick) were zero-loss imaged with the Zeiss LEO 912 EFTEM.

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