Endosome Sorting and Autophagy Are Essential for Differentiation and Virulence of *Leishmania major*®

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Cellular remodeling during differentiation is essential for lifecycle progression of many unicellular eukaryotic pathogens such as *Leishmania*, but the mechanisms involved are largely uncharacterized. The role of endosomal sorting in differentiation was analyzed in *Leishmania major* by overexpression of a dominant-negative ATPase, VPS4. VPS4E235Q accumulated in vesicles from the endocytic pathway, and the mutant *L. major* was deficient in endosome sorting. Mutant parasites failed to differentiate to the obligate infective metacyclic promastigote form. Furthermore, the autophagy pathway, monitored via the expression of autophagosome marker GFP-ATG8, and shown to normally peak during initiation of metacyclogenesis, was disrupted in the mutants. The defect in late endosome-autophagosome function in the VPS4E235Q parasites made them less able to withstand starvation than wild-type *L. major*. In addition, a *L. major* ATG4-deficient mutant was found also to be defective in the ability to differentiate. This finding, that transformation to the infective metacyclic form is dependent on late endosome function and, more directly, autophagy, makes *L. major* a good model for studying the roles of these processes in differentiation.

A screen for vacuolar protein sorting (Vps) mutants in yeast led to the discovery of the molecular machinery responsible for MVB formation and, in particular, the class E group of Vps mutants, which contained a large multimembrane cisternal compartment thought to represent an endosome unable to form intraluminal vesicles (3, 4). Vps class E proteins are organized in several sub-complexes named “endosomal sorting complexes required for transport” (ESCRT) I, II, and III (5–8). The ESCRT proteins are recruited from the cytoplasm to the endosomal membrane where they function sequentially in the formation of MVB vesicles and the sorting of proteins into the MVB pathway. It has been proposed that a multimeric AAA-type ATPase, Vps4, binds to ESCRT III and catalyzes the disassembly of this complex in an ATP-dependent manner (9). This Vps4-dependent dissociation of the ESCRT machinery is the final step of protein sorting into the MVB and is a prerequisite for vesicle formation (6). The Vps4 homologue in mammals, SKD1, is involved in membrane transport through endosomes and overexpression of a dominant negative mutant, SKD1E235Q, resulted in the production of aberrant endosomes defective in membrane transport between late endosomes and lysosomes (10).

In the protozoan parasite *Leishmania*, as in related kinetoplastid flagellates such as *Trypanosoma*, there is a complex membrane network highly polarized around the flagellar pocket, an invagination of the plasma membrane where the flagellum emerges from the cell body (11). This is an important zone of interaction between the parasite and its environment, because it is the only site in the cell for endocytosis and exocytosis and so is responsible for crucial exchanges such as uptake of nutrients via receptor-mediated endocytosis (12) and secretion of virulence factors that can interact with the host. Another characteristic of *Leishmania* parasites is that their promastigote (insect stage) form possesses a rather unusual lysosomal compartment, named the multivesicular tubule (MVT)-lysosome (13–15). This compartment has a low lytic capacity and a relatively high luminal pH in multiplicative procyclic promastigotes but acquires the properties of mature lysosomes as the parasite differentiates into its infective, but non-replicative, metacyclic form (13). So far, only a few components of the *Leishmania* endosomal machinery have been characterized at the molecular level such as early-endosomal Rab5 (16) and late-endosomal Rab7 (17). Indeed, although it has been shown that the MVT-lysosome is downstream of a MVB-like network of vesicular endosomes that surrounds the flagellar pocket (13), very little is known about them in *Leishmania*.

Among the pathways involving vesicular traffic in eukaryotic cells is autophagy, a process that is important for protein and organelle degradation during cellular differentiation and also as a defense against starvation conditions (18). The autophagic pathways of yeast and mammals have been characterized, and, although they have many common features, they appear to differ in some ways (19, 20). Central to the process in both is a vesicular compartment called the autophagosome, which is formed by a membranous structure that engulfs the cytoplasm/organelles that are to be degraded. The genesis of autophagosomal struc-

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2 The abbreviations used are: MVB, multivesicular bodies; VPS, vacuolar protein sorting; ESCRT, endosomal sorting complexes required for transport; MVT, multivesicular tubule; GFP, green fluorescent protein; PE, phosphatidylethanolamine; EF1α, elongation factor 1α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline.

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TABLE 1 Primers used in this study

| Primer             | Sequence (5'→3') |
|--------------------|------------------|
| OL1478             | 5’-CAATTCCTCACTGTACATTCCCACTTCGAC-3' |
| OL1479             | 5’-ACTTACATATAGTTGTATTCACTGCTAC-3' |
| OL1480             | 5’-CACATCATGTTTGTAGACTAGCTCCTCTGTC-3' |
| OL1481             | 5’-CACATGACGACATGCATTGTGTC-3' |
| OL1543             | 5’-CTCAGAGTCGATCTATGGTCCACGATGAGCTG-3' |
| OL1544             | 5’-CACCAGAATGCTACCTGACTGACCA-3' |
| OL1506             | 5’-AAGCTTCTAGATCTTTGTCGTCCTAGCGACTTCAATGTA-3' |
| OL1507             | 5’-GGCGTTCTGTGCTGGTGCAGCCCTCCTG-3' |
| NT158              | 5’-CAATTCCTCCCTCCACATAAGTT-3' |
| NT159              | 5’-CAACCAGTCTACAGTACCAGCAAA-3' |
| NT165              | 5’-AAGCTTCTAGATCTTTGTCGTCCTAGCGACTTCAATGTA-3' |
| NT168              | 5’-GTCGAGGTAGTATGTGGTCCGGGACGAGGACCAAGC-3' |
| NT170              | 5’-CCCGGTCAGATGTCTGCTGCTGCTAGCGCCCAA-3' |
| NT171              | 5’-AGCTTCATACAGACTGCTTACAGAGAAAGGGAC-3' |

OL1544 and cloned into BglII/Kpn1-digested PUS-containing pNUS-GFP-H vector (30). The resulting plasmid was named GFP-VPS4 and contained LmjVPS4 in-frame with the 3’-end of GFP. A plasmid (GFP-VPS4) containing LmjVPS4 was obtained by site-directed mutagenesis on plasmid GFP-VPS4, as described above. L. major ATG8 was amplified by PCR from genomic DNA with primers OL1506 and OL1507 and cloned into BglII/Xhol-digested pNUS-GFP-H vector to give GFP-ATG8.

Generation of L. major ATG4.2 Null Mutant and Re-expressing Cell Lines—The 1005-bp 5’-flank fragment of LmA T G4.2 (LmF30.03270) was generated by PCR from L. major genomic DNA with primers NT168 and NT169 (Table 1), digested with HindIII and SalI, and inserted into HindIII/Sall-digested pGL345HYG (31) to give pGL345ATG4.2-HYG5. The 3’-fragment was generated by PCR using primers NT170 and NT171. The resulting 1104-bp fragment was digested with Smal and BglII and cloned into pGL345ATG4.2-HYG5 to give pGLATG4.2-HYG53’. The cassette used for transfection was released by HindIII/BglII digestion. pGLATG4.2-BSD53’ plasmid, used for the replacement of the second ATG4.2 allele, was generated from plasmid pGLATG4.2-HYG53’ by replacing the SpeI/BamHI cassette containing the hygromycin resistance gene by a SpeI/BamHI cassette containing the blasticidin S deaminase gene. A population of parasites resistant to hygromycin was generated after transfection with pGLATG4.2-HYG53’. This population was used for a second round of transfection with the pGLATG4.2-BSD53’ construct. Two blasticidin-resistant clones from independent transfection events, designated Δatg4.2A1 and Δatg4.2B4, were selected for analysis.

For the re-expression experiment, the ATG4.2 gene, modified with a poly-histidine tag at its C-terminal end, was inserted into the pNUS epsomal vector. PCR using the primers NT158 and NT159 produced the poly-histidine-tagged version of ATG4.2. The resulting 1185-bp fragment was digested by NdeI/Xhol and ligated into pNUS-HnN plasmid (30), previously digested by the same enzymes, to give the pN-ATG4.2 plasmid. L. major wild-type promastigotes were electroporated with 15 μg of the episome, and transfectedants were selected with the appropriate antibiotic (Geneticin, G418, Invitrogen).

Complement-mediated Lysis and Macrophage Infections—The sensitivity of procyclic and metacyclic promastigotes to complement lysis was assessed with a protocol modified from (32). Briefly, 107 parasites were incubated at 37 °C in PBS mixed with an appropriate volume of fresh normal human serum. After 20 min, the samples were diluted 2-fold in PBS. The percentage of lysis, assessed by loss of promastigote motility and morphological changes, was calculated relative to control samples incubated in heat-inactivated serum, in which all cells remained viable.

Peritoneal macrophages from CD1 mice were adhered overnight in RPMI medium (Sigma) at 37 °C in 5% CO2/95% air on eight-chamber
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tissue culture plastic slides (Labtech) and then infected using late stationary phase promastigotes or peanut lectin-purified metacyclics at a ratio of 10 promastigotes per macrophage. After incubation for 3 h at 35 °C in 5% CO₂/95% air, non-phagocytosed promastigotes were removed by washing gently with RPMI four times, and the cultures were then incubated at 35 °C in 5% CO₂/95% air. The initial uptake of promastigotes by macrophages and their subsequent intracellular survival and growth as amastigotes was determined by counting the number of infected macrophages and the number of intracellular parasites in stained slides 3, 24, and 120 h after infection. Slides were fixed in methanol and stained with Giemsa to identify the parasites. The number of L. major per 100 macrophages was determined by examination of ~200 macrophages per assay.

Monitoring Autophagy—Live promastigotes of wild-type or VPS4-expressing L. major expressing GFP-ATG8 were observed daily by fluorescence microscopy, and the proportion of autophagosome-bearing cells as well as the number of these structures per cell were assessed. At least four series of 200 cells were counted per experiment for each point. To inhibit the formation of autophagosomes, promastigotes were grown with either 10 μM wortmannin (Sigma, 1 mM stock solution in Me₂SO) or 10 mM 3-methyladenine (Sigma, 30 mg/ml stock solution in water). To investigate responses to starvation conditions, promastigotes from early log phase cultures were sedimented by centrifugation and washed twice in PBS before being resuspended in pre-warmed PBS and incubated at 25 °C for up to 2 h.

Parasite Viability Assay—Viability of L. major during starvation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay adapted for Leishmania species (33). Briefly, 5 × 10⁶ promastigotes were starved in PBS as described above and then incubated for 45 min at 37 °C with MTT to a final concentration of 1 mg/ml, and the absorbance was measured at 620 nm using a microtiter plate reader. Control cells were grown in complete medium during the same period of time and similarly assessed with MTT. The results were expressed as a fraction of the values obtained for the starved cells relative to the values of the control cells (in percentages).

Antibodies and Immunoblotting—Anti-LmjVPS4 antibodies were raised in a rabbit using a peptide comprising the 15-amino acid C-terminal sequence (residues 361–375: CHFKRVVGPDPHDPTR). 10 μg of the peptide was expected to cause a defect in ATP hydrolysis (9). The wild-type parasite and cell line containing pXG-VPS4 showed morphological peculiarities and became broader and shorter (for example see GFP-fused VPS4E235Q in Fig. 2B, right). The use of an episomal vector for the expression of the VPS4 gene allowed us to increase expression levels with higher amounts of selective drug. This way we have shown that the growth defect was even more pronounced when the pXG-VPS4E235Q mutant was grown in higher concentrations of G418 (Fig. 1A, lower panel), with growth being hindered even in early log phase in culture, an increasing number of promastigotes expressing pXG-VPS4 and VPS4E235Q. The wild-type parasite and cell line containing pXG-VPS4 grew normally as promastigotes in vitro, but the pXG-VPS4E235Q mutant showed a premature exit from exponential growth phase. Indeed, the promastigotes overexpressing pXG-VPS4E235Q grew similarly to wild-type parasites throughout most of the logarithmic phase of growth in vitro but went into stationary phase at a lower density than wild-type or pXG-VPS4 promastigotes (Fig. 1B). During this growth phase in culture, an increasing number of promastigotes expressing VPS4E235Q showed morphological peculiarities and became broader and shorter (for example see GFP-fused VPS4E235Q in Fig. 2B, right). The use of an episomal vector for the expression of the VPS4 gene allowed us to increase expression levels with higher amounts of selective drug. Thus, we have shown that the growth defect was even more pronounced when the pXG-VPS4E235Q mutant was grown in higher concentrations of G418 (Fig. 1A, lower panel), with growth being hindered even in early log phase, and this correlated with increased amounts of VPS4E235Q protein at the higher G418 concentrations (Fig. 1A, lower panel inset). In contrast, cells transfected with the empty vector (pXG cell line) could withstand concentrations of G418 up to 50 μg/ml with little or no effect on growth (Fig. 1A, upper panel), confirming that neither the vector nor the resistance marker per se were toxic to the cells. Moreover, overexpression of VPS4 was shown not to be toxic to the cells as the pXG-VPS4 cell line grew normally in the presence of increased amounts of G418 and only showed a slight growth defect at 50 μg/ml (Fig. 1A, middle panel).

Localization of VPS4 and VPS4E235Q—L. major promastigotes were transfected with N-terminal GFP fusions of VPS4 and VPS4E235Q and
expression of the proteins confirmed with anti-VPS4 antibody (Fig. 2A, right panel, arrowhead) and anti-GFP antibody (data not shown). GFP-VPS4E235Q was apparently expressed at a lower level than GFP-VPS4, but, because the expression of GFP-VPS4E235Q was detrimental to Leishmania promastigotes (Fig. 1A), it is possible that the cells downregulated the protein to compensate for this effect. Live cells from these populations were observed by fluorescence microscopy (Fig. 2B).
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VPS4 showed a diffuse pattern throughout the cytosol, a similar localization to the GFP-fused SKD1 of mammals (10, 38) and HA-tagged Vps4 in yeast (9). However, the labeling obtained with GFP-VPS4E235Q was concentrated in structures (Figs. 2 B and 3) with shape and size similarities to the class E compartment of yeast, which is composed of exfoliated endosome-like organelles (3). The majority of these GFP-VPS4E235Q-containing structures in Leishmania promastigotes were present in the anterior part of the cell body, the location of the endocytic pathways in the parasite (16, 17).

To further characterize the compartment in which GFP-VPS4E235Q accumulated in Leishmania, co-localization experiments were performed with markers of endosomal compartments. Tomato lectin, which recognizes poly-N-acetyllactosamine of glycan-containing proteins localized in early endosomes of Trypanosoma brucei (39), was visualized close to GFP-VPS4E235Q but was clearly distinct (Fig. 2C, left panels). Concavalin A, which had previously been shown to co-localize with Rab7-endosomes in Leishmania (17), accumulated in the flagellar pocket before being internalized to reach vesicles (presumably Rab7-containing late endosomes) next to the nucleus. When left for 2 h to be internalized, concavalin A showed a partial co-localization with GFP-VPS4E235Q-positive compartments present in the anterior part of the cell (Fig. 2C, right panels). This suggests that the compartment in which GFP-VPS4E235Q accumulates is either part of the endosomal system or closely associated with it. This is fully consistent with VPS4 acting in a late endosomal localization as described for other organisms (9, 10, 38, 40).

Transport of Cargo to the Leishmania MVT-lysosome Is Defective in the VPS4E235Q Cell Line—The lipophilic dye FM4-64 was used to assess the integrity of transport to the lysosomal compartment in the pXG-VPS4E235Q promastigote line. This fluorescent endocytic marker is delivered to the vacuolar membrane in yeast (41) and has been used to identify class E compartments (4). Similarly, FM4-64 has been used in Leishmania as a marker for the MVT-lysosome in the stationary-phase promastigote form of the parasite (13). When used in a time-course experiment, the dye was found initially to be primarily labeling the flagellar pocket and the surface of the parasite (Fig. 3A, 0'). After a chase of 15–90 min, FM4-64 in the GFP-VPS4 line was apparent in the MVT-lysosome as well as the flagellar pocket (Fig. 3A, IS'–90'). In contrast, FM4-64 remained largely in the flagellar pocket in the GFP-VPS4E235Q line but also after the 90-min chase was observed in compartments associated with GFP-VPS4E235Q (Fig. 3A, 90', merged image). Additionally, we used fluorescent dextran as a fluid-phase endocytic tracer, because dextrans are known to be trafficked through the endosomal system to the MVT-lysosome of Leishmania (15). Dextran-red was trafficked normally to the MVT-lysosome in the pXG-VPS4-expressing cell line, whereas it accumulated in the VPS4E235Q-positive compartments in the mutant cells (Fig. 3B). These results indicate that there is possibly a defect in transport to the MVT-lysosome, or that there could be an alteration of that compartment, in the VPS4E235Q mutant. Furthermore, the fact that the FM4-64 signal did not co-localize with GFP-VPS4E235Q until 90-min incubation is in contrast with the lysosome already being labeled after 15 min in the control cell line. This suggests that the kinetics of internal vesicular transport are altered in VPS4E235Q-expressing parasites.

VPS4E235Q Mutants Fail to Differentiate into the Infective Metacyclic Form—Metacyclogenesis is crucial for the survival and pathogenesis of Leishmania in the mammalian host (42). Because the pXG-VPS4E235Q cell line was observed to prematurely enter stationary phase of growth during in vitro cultivation (Fig. 1B), we assessed if metacyclogenesis was affected in this cell line. Several properties distinguish the procyclic and metacyclic promastigote forms of L. major, including morphology (43), agglutination with peanut lectin (28), expression of stage-specific proteins HASPB or SHERP (Fig. 4A), uptake and delivery of FM4-64 to the MVT-lysosome was impaired in cells expressing GFP-VPS4 or GFP-VPS4E235Q (green), Arrowheads point co-localized signal. Scale bar = 10 μm.

VPS4E235Q Mutant Parasites Have a Defect in Autophagy—Endosomal membrane trafficking mediated by Vps4/SKD1 has been proposed to be involved in the late stages of the autophagic pathway in mammals (23) and also in regulating autophagy in yeast (24). Several orthologues of yeast proteins that have been implicated in the Atg8 lipidation pathway involved in autophagosome expansion and completion can be iden-
tified in *L. major*, including Atg3, two putative Atg4 cysteine peptidases, and several families of Atg8 proteins (44). The *L. major* ATG8 orthologue (*LmjF19.1630*), with the highest sequence identity to yeast Atg8 proteins was used in this study. ATG8 has been a useful marker for visualizing autophagosomes in yeast and mammalian cells (19, 45).

Thus, to track formation of autophagosomes in *L. major* we expressed ATG8 fused with GFP at its N terminus.

In log phase *L. major* promastigotes, GFP-ATG8 was distributed throughout the cytoplasm in most cells (Fig. 6A, top). However, in some early stationary phase promastigotes, autophagosomes could be identified as punctate structures clearly observable in the cytoplasm (Fig. 6A, center). To evaluate the extent of the variation in the occurrence of autophagosomes among stationary phase promastigotes, we expressed GFP-ATG8 and tracked autophagosome formation over time (Fig. 6B, bottom). The percentage of non-agglutinated cells was determined by the peanut agglutinin method. Values shown are the mean ± S.D. of three independent experiments.
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During stress by starvation, pXG-VPS4E235Q promastigotes died significantly quicker than did the pXG-VPS4 promastigotes (Fig. 7D), showing that the mutant is more sensitive to nutrient deprivation than the control cell line. Consistent with this, the presence of autophagosomes steadily increased during starvation of pXG-VPS4 promastigotes, whereas the number of pXG-VPS4E235Q promastigotes with GFP-ATG8-containing autophagosomes, which was initially higher in the controls, did not increase (Fig. 7E). These data suggest that the pXG-VPS4E235Q promastigotes have an autophagy defect, in which autophagosomes are not processed, and so accumulate, which makes the cells more sensitive to nutrient deprivation.

An ATG4 Mutant Has a Defect in Autophagy—To further assess if there is a direct involvement of autophagy in the cellular differentiation process, we produced a mutant of autophagy-related gene ATG4.2 (LmjF30.0270), which is one of two ATG4 homologues present in the genome of L. major (44). ATG4 is a cysteine peptidase involved in ATG8 processing and is crucial for autophagosome function (47, 48).

To investigate the involvement of VPS4 in autophagy in Leishmania, GFP-ATG8 was transfected into pXG-VPS4 and pXG-VPS4E235Q promastigotes. Log phase pXG-VPS4 promastigotes were similar to wild-type parasites in that, in most cells, GFP-ATG8 was found evenly distributed throughout the cytoplasm (Fig. 7A, left), whereas a few cells contained autophagosomes in addition to the evenly stained cytosol (Fig. 7A, center). Promastigotes expressing pXG-VPS4E235Q more frequently contained autophagosomes (Fig. 7A, right). Indeed, when analyzed for their production of autophagosomes, the pXG-VPS4 promastigotes showed a profile similar to that of wild type parasites (Fig. 7B, compare with Fig. 6B). In contrast, autophagosomes were more evident in the pXG-VPS4E235Q mutants even in early-log phase promastigotes, and the number of autophagosomes per positive cell was also found to be higher than in the pXG-VPS4 cell line (Fig. 7B).

Autophagy is induced in yeast and mammalian cells as a survival response under starvation conditions (18, 21). We found that the occurrence of autophagosomes in the pXG-VPS4 cells of L. major could be induced by starving early log phase promastigotes in PBS for 2 h (Fig. 7B). In contrast, the pXG-VPS4E235Q mutants, which initially displayed a higher number of autophagosomes than the pXG-VPS4 cell line, showed no increase in the number of autophagosomes under the same stress conditions (Fig. 7B). ATG8 undergoes several post-translational processing events resulting in conjugation to phosphatidylethanolamine (PE) and recruitment to the autophagosomal membrane (46). We used Western blotting analysis with anti-GFP antibody on cell extracts from pXG-VPS4 and pXG-VPS4E235Q cells expressing GFP-ATG8 to detect the membrane-associated form GFP-ATG8-PE (Fig. 7C). For the pXG-VPS4 cell line, the proportion of GFP-ATG8-PE increased during progression from log phase to early stationary phase of growth, or when cells were starved for 2 h in PBS. In contrast, the pXG-VPS4E235Q mutant displayed a higher level of the conjugated form both in log and early stationary phase. This is consistent with the data obtained by fluorescence observation of the GFP-ATG8 puncta (autophagosomes) in the same cell lines (Fig. 7B).

To determine if the autophagy defect of the pXG-VPS4E235Q promastigotes could be prevented by the addition of 3-methyladenine or wortmannin, inhibition, which was identified as the MVT-lysosome by co-localization of cells were observed with GFP-ATG8 labeling in a tubular compartment, the number of autophagosomes per cell and the percentage of cells with autophagosomes rapidly decreased. Concomitantly, a larger proportion of cells was observed with GFP-ATG8 labeling in a tubular compartment, which was identified as the MVT-lysosome by co-localization studies with FM4-64 (Fig. 6A, lower panel). The appearance of GFP-ATG8 positive autophagosomes in L. major promastigotes could be prevented by the addition of 3-methyladenine or wortmannin, inhibitors of autophagosomes formation in yeast and mammalian cells (19), to the culture medium (Fig. 6C).

To investigate the involvement of VPS4 in autophagy in Leishmania, GFP-ATG8 was transfected into pXG-VPS4 and pXG-VPS4E235Q promastigotes. Log phase pXG-VPS4 promastigotes were similar to wild-type parasites in that, in most cells, GFP-ATG8 was found evenly dis-
The ability of *L. major* Δatg4.2 null mutants to form autophagosomes was assessed by transfecting them so that they expressed the GFP-ATG8 marker. The proportion of Δatg4.2 promastigotes with GFP-ATG8-containing autophagosomes and the number of autophagosomes in each cell was higher than observed for wild-type cells at a similar stage of growth (Fig. 8B and data not shown). In addition, the number of autophagosomes did not decrease during stationary phase of growth, as observed with wild-type cells. Western blot analysis using anti-GFP antibody on cell extracts of early stationary phase wild-type and Δatg4.2 promastigotes expressing GFP-ATG8 showed that the mutant cell line had an increased proportion of the lipidated form of GFP-ATG8 relative to the cleaved form (Fig. 8B). This result is consistent with an accumulation of the membrane-bound conjugated form of GFP-ATG8 in Δatg4.2 promastigotes.

As with the VPS4 mutant, stress by starvation was tested on the Δatg4.2 promastigotes. Similarly, Δatg4.2 promastigotes were also less able to withstand starvation than the wild-type cells (Fig. 8C), suggesting that the accumulated structures bearing GFP-ATG8, designated as autophagosomes, are unable to proceed along the autophagy pathway, which results in an autophagy defect. This phenotype could be restored almost to wild-type levels by episomal re-expression of a copy of ATG4.2 in the mutant (Δatg4.2[pN-ATG4.2]) (Fig. 8C).

**FIGURE 6.** Autophagosome formation in wild-type *L. major* promastigotes. A, distribution of GFP-ATG8 in *L. major* promastigotes. Arrowheads indicate the autophagosomes. Late stationary phase promastigotes were labeled with FM4-64 and its co-localization with GFP-ATG8 assessed using fluorescent microscopy. Scale bar = 10 μm. In B, Upper panel, occurrence of GFP-ATG8-associated autophagosomes and the MVT lysosome in *L. major* promastigotes. The proportion of GFP-expressing cells with autophagosomes or tubular-shaped GFP signal was assessed. The corresponding growth curve is shown in dashed line with corresponding scale on the right. Lower panel: average number of autophagosomes per promastigote containing autophagosomes during growth in vitro. Data are means ± S.D. from three independent experiments. C, *L. major* promastigotes expressing GFP-ATG8 were grown in medium containing either 10 μM wortmannin (WM) or 10 mM 3-methyladenine (3MA) for 4 days (cell density prior to addition of the inhibitor was ~3 × 10⁶ cells/ml). The number of GFP-ATG8-associated autophagosomes was counted prior to the addition of the inhibitor (initial) and after 4 days. Promastigote multiplication was not significantly affected by treatment with inhibitors. Data are means ± S.D. from three independent experiments.
**FIGURE 7.** Autophagosome formation in the VPS4^{E235Q} line and survival during starvation. A, localization of GFP-ATG8 in live mid-log phase pXG-VPS4 and pXG-VPS4^{E235Q} promastigotes. Arrowheads indicate autophagosomes. Scale bar = 10 μm. In B: Upper panel, occurrence of GFP-ATG8 autophagosome-containing promastigotes during growth in vitro. Corresponding cell densities are as follows: early log (~5 × 10^5 parasites/ml), mid-log (~5 × 10^6 parasites/ml) or early stationary phase (~9 × 10^6 parasites/ml for pXG-VPS4^{E235Q}, ~2 × 10^7 parasites/ml for pXG-VPS4). The number of promastigotes containing at least one GFP-ATG8 autophagosome is expressed as a percentage of all GFP-expressing cells. Lower panel, average number of autophagosomes per positive promastigote. Data are means ± S.D. from four series of measurements from at least two independent experiments. *, data for pXG-VPS4^{E235Q} cell line differs significantly from pXG-VPS4 cell line control (p < 0.01). C, Western blot analysis of GFP-ATG8-PE. Extracts from pXG-VPS4 and pXG-VPS4^{E235Q} promastigotes expressing GFP-ATG8 were analyzed by SDS-PAGE in the presence of 6 M urea and Western blotting with an anti-GFP antibody detecting both GFP-ATG8 and GFP-ATG8-PE. Phosphatidylethanolamine (PE) could be removed by treatment of the extracts with 10 units of phospholipase D (PLD) at 37 °C for one hour prior to analysis (right-hand lane). EF1α serves as an internal loading control. D, sensitivity to starvation. Log phase promastigotes were incubated in PBS, and the viability of the cells was assessed by the MTT assay. Data are means ± S.D. from four replicates. *, data for pXG-VPS4^{E235Q} cell line differed significantly from pXG-VPS4 cell line control (p < 0.01). E, proportions of GFP-ATG8 autophagosome-containing cells during starvation conditions. Promastigotes were treated as in D, and the number of GFP-ATG8-containing cells was assessed by microscopic observation. Data are means ± S.D. from four series of data from two independent experiments.
**FIGURE 8.** \( \Delta \text{atg}4.2 \) is defective in autophagy. **A**, schematic representation of the ATG4.2 locus and the plasmid constructs used for gene replacement. The ATG4.2 and antibiotic resistance genes are shown as arrows, flanking DNA sequences are shown as boxes. Restriction enzymes used for the different constructs and the expected sizes of the Ndel/BglII fragments are shown. 5'-DHPR and 3'-DHPR, dihydrofolate reductase flanking regions; BSD, blasticidin resistance gene; HYG, hygromycin resistance gene. On the right is shown the Southern blot analysis of genomic DNA digested with Ndel and BglII, separated on a 1% agarose gel, blotted onto a nylon membrane and hybridized with a \( ^{32} \text{P} \)-labeled DNA probe corresponding to the 5'-flanking region of ATG4.2. Molecular size markers are shown on the left (kb). **B**, occurrence of GFP-ATG8 autophagosomes in \( \Delta \text{atg}4.2 \) and wild-type L. major promastigotes. Left, the proportion of GFP-expressing cells with autophagosomes during growth was assessed. The corresponding growth curve is shown in Fig. 6B. Data are means ± S.D. from three independent experiments. Right, a Western blot analysis of extracts from early stationary phase wild-type and \( \Delta \text{atg}4.2 \) promastigotes expressing GFP-ATG8, after SDS-PAGE containing 6 M urea and with an anti-GFP antibody to detect GFP-ATG8 and GFP-ATG8-PE. EF1\( \alpha \) serves as an internal loading control. **C**, sensitivity to starvation. Log phase promastigotes were incubated in PBS, and the viability of the cells was assessed by the MTT assay. Data are means ± S.D. from three independent experiments. *, data for \( \Delta \text{atg}4.2 \) cells differed from other cell lines (\( p < 0.01 \)).
The ATG4 Mutant Fails to Differentiate into the Infective Metacyclic Form—We assessed the ability of \( \Delta \text{atg}4.2 \) promastigotes to differentiate into metacyclic forms using the same criteria as pXG-VPS4E235Q cells. Stationary phase \( \Delta \text{atg}4.2 \) were found to be defective in expression of stage-specific metacyclic proteins HASPB and SHERP (Fig. 9A) and were more sensitive to lysis by human serum (Fig. 9B). One difference between the pXG-VPS4E235Q cells and \( \Delta \text{atg}4.2 \) was their agglutination by peanut lectin (Fig. 9C). Whereas ~30% of wild-type and pXG-VPS4E235Q cells were agglutinated by peanut lectin, only 5–10% of \( \Delta \text{atg}4.2 \) promastigotes were agglutinated, showing some phenotypic variance between the two different mutant cell lines. All these phenotypes were similar to wild type in the \( \Delta \text{atg}4.2[p\text{N-ATG}4.2] \) mutant (Fig. 9). These data show that \( \Delta \text{atg}4.2 \) promastigotes are defective in metacyclogenesis.

**DISCUSSION**

In this study we have investigated the properties of \( L. \text{major} \) MVB by creating dominant-negative mutants of VPS4, which disrupt MVB sorting function. In yeast, Vps4 is involved in the disassembly of the ESCRT III complex from the endosomal membrane, and the ATP hydrolysis mutant cannot dissociate from the late endosomal membrane (9). In \( L. \text{major} \), GFP-VPS4E235Q was localized to a vesicular compartment, which partially co-localized with the late endosomal marker concanavalin A at the anterior end of the cell, between the flagellar pocket and the nucleus, whereas GFP-VPS4 was found throughout the cytosol (Fig. 2B). This is consistent with VPS4 having a similar role in \( L. \text{major} \) as in yeast and mammalian cells, and this is further supported by the finding of genes encoding other components of the ESCRT III complex in the...
genome of *L. major* (supplementary Table SI). It has been proposed for the mammalian SKD1 mutants that these abnormal endosomes might represent a hybrid organelle between late endosomes and lysosomes (49). Our data are consistent with a close association of VPS4E235Q with endosomal structures. Additional experiments with FM4-64 and the fluid phase marker dextran-red confirmed that in the VPS4E235Q mutant endocytosed cargo could not reach the MVT-lysosome. However, the cellular distribution and expression levels of glycosylphosphatidylinositol-anchored surface peptide GP63 remained unchanged in the mutant (data not shown), showing that there were no major defects in exocytosis in the mutants. The impairment in lysosomal transport is similar to the situation observed for yeast and mammalian Vps4/SKD1 mutants (9, 49). This impairment in *L. major*, however, caused a severe growth phenotype resulting in cells that lacked many established markers of metacyclic promastigotes, showing that they had a defect in metacyclogenesis. The one exception was that the VPS4E235Q cells expressed metacyclic-specific lipophosphoglycan on their cell surface. Lipophosphoglycan assembly is complex and necessitates biosynthetic steps in the Golgi, before being transported to the surface (50), so it is possible that this biosynthetic pathway is induced independently of metacyclic-specific proteins such as SHERP and HASP and earlier in the process of metacyclogenesis. One of the consequences of this inability to fully differentiate into the metacyclic form was reduced infectivity and survival of the mutant parasites in macrophages.

In yeast and mammalian cells, late endosomal membrane trafficking mediated by Vps4/SKD1 have been shown to be important for the function of the autophagic pathway (23, 24). In *Leishmania*, autophagy, monitored by the presence of GFP-ATG8-positive autophagosomes, was most active during the differentiation of *Leishmania* procyclic promastigotes to the infective metacyclic promastigote form (Fig. 6). It also could be induced in procyclic promastigotes by starving the cells (Fig. 7, B and D), a stimulus that induces autophagy in yeast and mammals. Moreover, the appearance of the structures could be prevented by the use of known autophagy inhibitors wortmannin and 3-methyladenine (19), thus further confirming their autophagosomal nature.

In VPS4E235Q cells, the number of GFP-ATG8-positive cells and autophagosomal structures within them was higher than in the VPS4 control, both in log phase and stationary phase cells. However, they did not increase over a certain limit, suggesting that there is some feedback signal that prevents further production of autophagosomes. Furthermore, the lipidated form of ATG8 (ATG8-PE) was found to be consistently present at higher levels in VPS4E235Q cells than in wild-type cells. This suggests that the VPS4E235Q cells accumulated autophagosomes, due to a defect in the ability of the autophagosomes to fuse with the endosomal/MVT-lysosomal compartment. In wild-type parasites, the GFP-ATG8 signal localized to the MVT-lysosome in metacyclic parasites (Fig. 6A). We propose that the differentiation between dividing procyclic promastigotes and the infective metacyclic form, which differ by many aspects of cell shape, metabolism, and surface proteins, is dependent on endosome function and autophagy. The functioning of the autophagic pathway in mammalian cells requires a functional MVB compartment as illustrated by the defect in autophagy in the Vps4 mutant (23). However, the extent of the involvement of the endosomal compartment in the autophagic pathway in yeast is less clear (20, 51).

We have now clearly shown that in *Leishmania*, the VPS4E235Q cells had a defect in autophagy, and we observed that VPS4-positive structures were located close to autophagosomal structures in the cell (data not shown), suggesting that in this lower eukaryote the autophagic pathway could require interaction with the endosomal network in order to function.

More direct evidence for the involvement of autophagy in differentiation of the parasite was provided by the phenotype of the ATG4.2 null mutants. ATG4 is essential for autophagy in both yeast and mammals (47, 48); it is a cysteine peptidase that is involved in the regulation of the autophagosomal membrane association of ATG8 by the conjugation/deconjugation of the protein to PE. In yeast, where there is only one isoform of ATG4, the ATG4 mutant loses the ability to both conjugate and deconjugate ATG8, leading to a defect in autophagy (46). There are two ATG4 isoforms in mammalian cells, but their respective substrates and specific roles have not yet been clearly elucidated (52, 53). We report here that the null mutant of one of the two *L. major* ATG4 genes (Δatg4.2) accumulates autophagosomes and has a defect in autophagy, as assessed by the reduced ability of the mutants to withstand starvation. It leads us to speculate that ATG4.2 might be involved in ATG8 deconjugation prior to fusion with late endosomes/lysosomes, a process that is necessary for efficient progression of autophagy in yeast (46). The Δatg4.2 mutant is also impaired in its ability to differentiate into the metacyclic form, thus there is a clear link between the autophagic machinery and the differentiation process. It is notable that for most markers of metacyclogenesis VPS4E235Q and Δatg4.2 were similar, however, the latter did not express metacyclic-specific lipophosphoglycan on its cell surface. This could highlight a different role between endosome sorting and autophagy in the completion of differentiation.

The VPS4E235Q cells were more sensitive to starvation than the control promastigotes and, unlike the control cells, did not increase the number of autophagosomes in this condition (Fig. 7). It is interesting to note that previous work done on csc1/Vps4 in *Saccharomyces cerevisiae* had shown that starvation-induced autophagy was impaired in a null mutant of this gene (24). Thus, VPS4 mutants do not seem to be affected in their ability to initiate the formation of ATG8-bearing autophagosomes but rather accumulate these structures.

The increased sensitivity of the VPS4E235Q cells to starvation could be a combination of several defects. Firstly, the VPS4E235Q line appeared defective in the transport of endocytosed cargo to the MVT-lysosome, and thus its ability to acquire and transfer nutrients from the medium would be compromised. However, the MVT-lysosome itself does not appear to be significantly altered, because the CBP lysosomal cysteine peptidase is correctly trafficked, processed, and activated in the MVT-lysosome in VPS4E235Q cells (data not shown). Secondly, defects in the final stages of the autophagic pathway in VPS4E235Q cells make the parasites more sensitive to starvation conditions due to a reduced ability of the parasites to recycle, as reserve nutrients, cytoplasmic or organelar material. This latter defect is likely to be important, because similar results were found when the ATG4.2 mutant was put in similar conditions (Fig. 8), directly highlighting the role of autophagy in the survival of *Leishmania* during starvation.

Starvation conditions and high population density are strong inducers of autophagy in both yeast and mammalian cells (18, 21). In those conditions, autophagy has been proposed to be associated with stress-induced cell differentiation (18), where it could help remodel cell shape. This notion was until recently not supported by consistent experimental evidence, but a few examples of such a role of autophagic processes in differentiation have now emerged (25–27). *Leishmania* promastigotes reach an *in vitro* stationary phase of growth where their cell density is at a peak, and it is at this stage that they differentiate from dividing promastigote to the infective metacyclic form. We observed an increased number of autophagosomes at the flagellar membrane and in the cytosol of the parasites to recycle, as reserve nutrients, cytoplasmic or organelar material. This latter defect is likely to be important, because similar results were found when the ATG4.2 mutant was put in similar conditions (Fig. 8), directly highlighting the role of autophagy in the survival of *Leishmania* during starvation.

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While most autophagy studies made so far have focused on the role of autophagy during stress-induced cellular remodeling, we have shown for the first time that autophagy has an important role in the differen-
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tiation and acquisition of virulence of a protozoan parasite. Proposed roles for autophagy extend from nutrient acquisition and cellular remodeling to growth control, but its role in differentiation is essentially unexplored. Full characterization of the molecular mechanisms of autophagy, which are largely conserved from unicellular eukaryotes to metazoans, but probably differ in detail through evolutionary adaptation, can greatly benefit from the study of Leishmania, which has distinct morphological forms and can be genetically manipulated.

Acknowledgements—We are indebted to Emmanuel Tetaud for the gift of his pNUS vector. Thanks to Deborah Smith for the gifts of anti-HASPB and anti-SHERP antisera and to the Wellcome Trust Sanger Institute for L. major genomic sequence data.

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