Distinct Roles in Autophagy and Importance in Infectivity of the Two ATG4 Cysteine Peptidases of *Leishmania major*

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**Background:** ATG4 is a cysteine peptidase crucial for macroautophagy.

**Results:** Gene deletion mutants show that the two ATG4s of *Leishmania* perform distinct roles, although there is some redundancy.

**Conclusion:** ATG4s are not individually essential but macroautophagy, a process important in the virulence of the parasite, requires one.

**Significance:** Highlights the distinct roles of ATG4 isoforms and their importance for autophagy and parasite infectivity.

Macroautophagy in *Leishmania*, which is important for the cellular remodeling required during differentiation, relies upon the hydrolytic activity of two ATG4 cysteine peptidases (ATG4.1 and ATG4.2). We have investigated the individual contributions of each ATG4 to *Leishmania major* by generating individual gene deletion mutants (Δ*atg4.1* and Δ*atg4.2*); double mutants could not be generated, indicating that ATG4 activity is required for parasite viability. Both mutants were viable as promastigotes and infected macrophages in vitro and mice, but Δ*atg4.2* survived poorly irrespective of infection with promastigotes or amastigotes, whereas this was the case only when promastigotes of Δ*atg4.1* were used. Promastigotes of Δ*atg4.2* but not Δ*atg4.1* were more susceptible than wild type promastigotes to starvation and oxidative stresses, which correlated with increased reactive oxygen species levels and oxidatively damaged proteins in the cells as well as impaired mitochondrial function. The antioxidant N-acetylcysteine reversed this phenotype, reducing both basal and induced autophagy and restoring mitochondrial function, indicating a relationship between reactive oxygen species levels and autophagy. Deletion of *ATG4.2* had a more dramatic effect upon autophagy than did deletion of *ATG4.1*. This phenotype is consistent with a reduced efficiency in the autophagic process in Δ*atg4.2*, possibly due to ATG4.2 having a key role in removal of ATG8 from mature autophagosomes and thus facilitating delivery to the lysosomal network. These findings show that there is a level of functional redundancy between the two ATG4s, and that ATG4.2 appears to be the more important. Moreover, the low infectivity of Δ*atg4.2* demonstrates that autophagy is important for the virulence of the parasite.

Macroautophagy (hereafter autophagy) is an intracellular process that sequesters cytosol and organelles within autophagosomes for delivery to and degradation within lysosomes. One main physiological significance of this process in most cell types is recycling of intracellular materials during nutrient deprivation, but in addition it plays important parts in cellular differentiation, tissue remodeling, growth control, size regulation, mitochondrial homeostasis, cellular immunity, adaptation to stresses, and unconventional protein secretion (1–6). The many steps comprising autophagy rely on a large repertoire of proteins mainly from the ATG family, of which more than 30 have been described. Central to the process are the ATG5-ATG12 conjugation and ATG8-lipidation pathways, which are coordinated by the activity of the ATG6-Vps34 complex (7–9).

ATG4, a cysteine peptidase of Clan CA, family C54, is a key component of the ATG8-lipidation pathway and is essential for autophagosome biogenesis. ATG4 is responsible for cleavage of ATG8 after a glycine residue close to its C terminus, facilitating conjugation of ATG8 to the lipid phosphatidylethanolamine (PE). ATG8-PE attaches to the phagophore and is involved in elongation of the phagophore and closure of the autophagosome. Subsequently, ATG4 cleaves ATG8 from the PE on the outside of the autophagosome membrane, the reverse of the initial conjugation reaction (10–13), which allows interaction and fusion of the autophagosome with the endosomal-lysosomal compartments (14–16). The conjugation and deconjugation activities of ATG4 are crucial for autophagosome genesis and function.

Mammals and plants have multiple orthologues of the single yeast ATG4. There appears to be some functional redundancy (17) but the four ATG4 orthologues in humans have distinct roles in autophagosome genesis and cell survival, which is reflected in their preferred substrates among the six ATG8 orthologues. ATG4B has activity toward all of the ATG8 homologues and is the key regulator for autophagy in humans, its absence has severe consequences in the cell (18–21). ATG4D is predominantly involved in mitophagy and apoptosis (22, 23).

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2 The abbreviations used are: ATG/ATG, autophagy related gene/protein; H₂DCF-DA, 2,7 ″-dichlorodihydrofluorescein diacetate; PE, phosphatidylethanolamine; ROS, reactive oxygen species; NAC, N-acetylcysteine; SE, scanning electron microscopy.
whereas ATG4C has restricted substrate specificity and is only required for survival during prolonged stress (19).

Human ATG4A and ATG4B are redox regulated, they are the target for direct but reversible oxidation of a regulatory cysteine residue by reactive oxygen species (ROS), which inactivates the deconjugation activity (24, 25). The catalytic cysteine of ATG4 that processes ATG8 prior to its conjugation to PE is not susceptible to ROS oxidation. Its continued activity ensures that the lipidated ATG8 is formed and, in the absence of the deconjugation activity of ATG4, accumulates within the cell and is available for increased autophagosome biogenesis under conditions of oxidative stress and starvation. Moreover, ROS can up-regulate the expression of ATG4 in a JNK signaling-dependent manner to ensure that an adequate amount of processed ATG8 is available for autophagosome biogenesis after autophagic induction in MEF-7 cells (26). This up-regulation of ATG4 expression can be abrogated by either JNK knockdown or addition of the ROS scavenger N-acetylcysteine (NAC). The relationship between ROS and JNK activation was established previously (27, 28), but this link with ATG4 demonstrates a clear correlation between ROS production and the induction of autophagy. Oxidative stress caused by high ROS levels causes extensive and irreversible structural changes to proteins and organelles within cells (29, 30), thus the induction of autophagy is a mechanism to maintain quality control, cellular homeostasis, and cellular energetic balance (24). Further evidence of this link comes from the findings that autophagy-deficient mutants have high ROS levels. Human ATG4C and ATG4D are not redox regulated in the same way and neither are the ATG4s of yeast and plants, suggesting that these latter organisms must deal with ROS stress differently.

Leishmania, the causative agent of important diseases known as the leishmaniases, which are prevalent in many tropical and subtropical regions, carries out autophagy, comprising two conjugation pathways similar to yeast and higher eukaryotes, including the parts that they may play in dealing with ROS, the relationship between ROS and autophagy in Leishmania, and the importance of the ATG4s for parasite survival and infectivity.

**Experimental Procedures**

**Parasite Handling Procedures**—Promastigotes of L. major (MHOM/IL/80/Friedlin, designated WT for this study) were grown in modified Eagle’s medium (designated complete HOMEM medium) with 10% (v/v) heat-inactivated fetal calf serum at 25 °C, as described previously (34). Parasite numbers were estimated using an improved Neubauer hemocytometer. In this report, except when stated otherwise, early log, mid-log, and early stationary phases of promastigote growth correspond to ×5 × 10⁶, 5 × 10⁶, and ×9 × 10⁶ parasites ml⁻¹, respectively. The following antibiotics were added to the cultures of the Δatg4 mutants and the derived cell lines as follows: hygromycin B (Sigma) at 50 µg ml⁻¹; phleomycin (CaHy, France) at 10 µg ml⁻¹; puromycin (Calbiochem) at 10 µg ml⁻¹; blasticidin S (Calbiochem) at 10 µg ml⁻¹; and neomycin (G418; Geneticin, Invitrogen) at 25 µg ml⁻¹.

**Metacyclogenesis and Infectivity of Leishmania to Macrophages and Mice**—Metacyclic promastigotes in L. major stationary phase cultures were assessed by using either the peanut agglutinin method (35) or Western blot analysis using antibodies to the metacyclic promastigote-specific protein, HASPB (see below).

Infectivity of Leishmania lines to macrophages was determined using peritoneal macrophages from CD1 mice resuspended in RPMI media (Sigma) at 5 × 10⁵ ml⁻¹ and left to adhere onto coverslips overnight. These cells were infected with stationary phase promastigote cultures or amastigotes harvested from mice footpad lesions at a ratio of ∼2 promastigotes or 0.5 amastigotes per macrophage and incubated for up to 5 days at 32 °C in 5% CO₂, 95% air. Non-phagocytosed promastigotes or amastigotes were removed after 24 h by washing four times with RPMI. Parasite abundance within the macrophages after 1 and 5 days was determined after the cells were fixed in methanol and stained with Giemsa for 10 min.

The infectivity to mice was determined using groups of 5 mice that were inoculated subcutaneously within a footpad with 5 × 10⁵ stationary phase L. major promastigotes or amastigotes, harvested from mice footpad lesions, suspended in 200 µl of PBS, pH 7.4. The thickness of the lesion in infected footpads was measured using a caliper over a 5–12-week period.

**Amastigote Isolation from Infected Mice and Transformation to Promastigotes**—Amastigotes were excised, into cold PBS to Promastigotes or amastigotes, harvested from mice footpad lesions, suspended in 200 µl of PBS, pH 7.4. The thickness of the lesion in infected footpads was measured using a caliper over a 5–12-week period.

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Amastigote Isolation from Infected Mice and Transformation to Promastigotes—Amastigotes were excised, into cold PBS containing 50 µg ml⁻¹ of gentamycin (Sigma), from footpad lesions of mice, inoculated 5 weeks earlier with 5 × 10⁵ stationary phase promastigotes, after removal of the skin. The lesion material was ground in a glass tissue grinder and centrifuged at 150 × g for 1 min at 4 °C to remove the large debris. The supernatant was then centrifuged at 1700 × g for 15 min and the subsequent pellet was resuspended and treated as required by the design of the experiment.

The number of L. major amastigotes in footpad lesions of infected mice was determined using the limiting dilution assay as described previously (36). Briefly, amastigotes of L. major were excised from footpads and resuspended in 10 ml of complete HOMEM medium with 50 µg ml⁻¹ of gentamycin. The parasite suspension was serially diluted in duplicate flasks, incubated for 5 days at 26 °C, and then inspected daily for parasite growth. For quantifying the morphological forms in these cultures, parasites within the culture flasks were classified according to the following criteria: amastigotes, ovoid to round but lacking an emergent flagellum, or promastigotes, elongated bodies with a flagellum equal to or longer than the cell body...
Leishmania ATG4 Cysteine Peptidases

length. Other parasites were designated as intermediate forms. A minimum of 200 cells was examined and the resulting differential counts were expressed as percentages.

Western Blot Analyses—Parasites were harvested at 1,000 × g for 10 min, washed twice in PBS, and the pellets were either used immediately or stored at −20 °C. Parasite lysates used for this analysis were produced by resuspension of parasite pellets in lysis buffer, comprising 0.25 M sucrose, 0.25% Triton X-100, 10 mM EDTA, and a mixture of peptidase inhibitors (10 μM E-64, 2 mM 1,10-phenanthroline, 4 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). After a 10-min incubation in ice, lysates were centrifuged at 15,700 × g for 30 min at 4 °C, and the resulting supernatant (designated soluble fraction) was retained for analysis. Protein concentrations were determined according to the Bradford procedure (Bio-Rad) using bovine serum albumin as the protein standard.

Generation of L. major ATG4 Null Mutants and Re-expressing Cell Lines—To generate the ATG4.1 null mutant, the 1014-bp 5′-flank fragment of ATG4.1 (LmjF32.3890) was amplified by PCR with primers NT164 (5′-CCC GGG TAC CAG GGC GGG GGT GTA G-3′) and NT165 (5′-GTC GAC TCT GGA GAT GCT GTA G-3′), digested with HindIII and SalI, and inserted into the HindIII/SalI-digested pGL345-HYG (37) to give pGL345ATG4.1-HYG5′. The 3′ flank fragment was amplified by PCR with primers NT166 (5′-CCC GGG TCG TAG CGC CAG GAC GGT GTA GCT GTA G-3′), digested with HindIII and SalI, and inserted into the HindIII/SalI-digested pGL345-HYG (37) to give pGL345ATG4.1-HYG5′. The cassette used for transfection of L. major promastigotes was released by HindIII/BglII digestion. The pGL345ATG4.1BDS5′ plasmid used for replacement of the second allele was generated from plasmid pGL345ATG4.1HYG5′ by replacing the SpeI/BamHI ORF of the hygromycin resistance gene with a SpeI/BamHI ORF of the histidine-tagged ATG4.2 but was amplified using primers NT160 (5′-CTC GAG ATG GCT CCG TGT AGA AGA TT-3′) and NT161 (5′-AGA TCT TCA ATG ATG ATG ATG ATG ATG ATC CAG CTA CTC CCA-3′). The resulting cell line was designated Δatg4.2::ATG4.2. The creation of cell lines expressing GFP-ATG8 and ATG4.2 have been described previously (33).

L. major promastigotes were electroporated (Nucleofector™) with 15 μg of DNA of the extra chromosomal constructs, namely pN-GFP-ATG8 and pN-ATG4.2, and 30 μg of the pRIB-PurATG4.1 or pRIB-PurATG4.2 cassettes. Transfectants were selected with the appropriate antibiotic at the concentrations detailed above. All PCR assays were carried out in a GeneAmp 9600 PCR system (PerkinElmer Life Sciences) for 30 cycles of denaturation (94 °C, 15 s), annealing (65 °C, 15 s), and extension (72 °C, 2 min). For the attempts to generate the ATG4 double null, the pGL345ATG4.1NEO5′ and pGL345ATG4.1BLE5′ plasmids were generated from plasmid pGL345ATG4.1HYG5′ by replacing the SpeI/BamHI ORF of the hygromycin resistance gene with SpeI/BamHI ORFs of the neomycin resistance and bleomycin resistance genes, respectively.

Southern Blot Analyses—Genomic DNA was extracted according to standard procedures (40). DNA (10 μg) was digested with XhoI, fractionated by agarose gel electrophoresis, nicked, denatured, neutralized, and blotted onto Hybond™N* membrane (Amersham Biosciences) by capillary transfer. The probe was prepared from a 1014-bp HindIII/Sall 5′ flank fragment excised from the T-vector (Promega) using a random priming kit (Prime-It; Stratagene) and purified on Microspin S-200 HR columns (Amersham Biosciences). Filters were hybridized overnight at 65 °C with a [α-32P]dATP-labeled 5′ flank fragment probe in Church-Gilbert hybridization solution. Filters were washed under high stringency (15 min at 65 °C with 2× SSC, 0.1% SDS and twice with 0.2× SSC, 0.1% SDS) and exposed to x-ray film (Konica Medical Film).

Monitoring and Quantification of Autophagy—For live imaging, promastigotes of WT[pN-ATG8], Δatg4.1[pN-ATG8], Δatg4.1b, were selected for analysis. The generation of the Δatg4.2 line has been described previously (33).

To generate the lines re-expressing ATG4.1, the ATG4.1 gene modified with a histidine tag at the C-terminal end (to verify protein expression) was inserted in the pRIB-Pur bearing the puromycin resistance gene (38). PCR primers NT158 (5′-CTC GAG ATG GGC ACG AAC GCC AAA GTG GCA GAG-3′) and NT159 (5′-ATC GAT CTA ATG ATG ATG ATG ATG GCT GCC TGG AGA AGA GAT TGA ATT-3′) produced the histidine-tagged version of ATG4.1 containing BglII and BamHI sites, respectively. The 1077-bp fragment was digested by BglII/BamHI and cloned into pRIB-Pur vector previously digested with the same restriction enzymes to give the pRIB-PurATG4.1 plasmid. The integration cassette from this plasmid was excised by digestion with Pael and Pmel before electroporation of L. major promastigotes. This fragment possesses the 5′ and 3′ flanking regions for chromosomal integration at the ribosomal locus. Promoters at these loci are constitutively expressed in both promastigote and amastigote life cycle forms (39). This cell line was designated Δatg4.1:ATG4.1. The pRIB-PurATG4.2 plasmid was made similarly using the histidine ATG4.2 but was amplified using primers NT160 (5′-CTC GAG ATG GCT CCG TCT CGT GCA AGA TT-3′) and NT161 (5′-AGA TCT TCA ATG ATG ATG ATG ATG ATC CAG CTA CTC CCA-3′). The resultant cell line was designated Δatg4.2::ATG4.2. The creation of cell lines expressing GFP-ATG8 and ATG4.2 have been described previously (33).
Leishmania ATG4 Cysteine Peptidases

Data Processing—Experimental data from macrophage infections, mice infectivity, Alamar Blue, MitoTracker Red, MitoTracker Green, and H$_2$DCF-DA assays were pooled for comparison using unpaired $t$ tests. A $p$ value of $<0.05$ was used as the level of significance. Counts of puncta from ATG-tagged proteins and the measurements of body lengths of promastigotes were performed on at least 100 cells and analyzed statistically as described above.

RESULTS

Targeted Deletion of the $L. major$ ATG4 Genes—We have described previously the generation of the ATG4.2 null mutant (Δatg4.2) and an add-back line (Δatg4.2::ATG4.2) and how analysis of the mutants demonstrated that the cysteine peptidase ATG4.2 is important for differentiation between developmental forms of *Leishmania* (33). To facilitate a comparison between the contributions of the two ATG4s in the biology of the parasite, we generated a ATG4.1 gene deficient cell line (Fig. 1A). The two independent clones obtained (designated Δatg4.1a and Δatg4.1b) were confirmed by Southern blot analysis to lack the ATG4.1 gene and have the correct integration of the replacement cassette (Fig. 1B). Hybridization of Xhol-digested genomic DNA with a 5′-flank probe revealed a 7.6-kb product corresponding to the ATG4.1 locus of the WT parasites, which is absent in the Δatg4.1 lines. In these, a DNA fragment of −5.0 kb is visible, consistent with correct integration of the HYG and BSD drug resistance markers in the ATG4.1 locus. As a control, ATG4.1 modified with a C-terminal His$_6$ tag was re-expressed in the Δatg4.1 lines (to give Δatg4.1::ATG4.1). Successful expression of ATG4.1 was confirmed by detection of a 43-kDa protein, which was absent from lysates of WT and Δatg4.1 promastigotes, by Western blot analysis with anti-His antibody (Fig. 1C). Subsequent phenotypic analyses were performed on both Δatg4.1 clones, but as they behaved similarly data are presented for Δatg4.1a only (hereafter referred to as Δatg4.1). Although the Δatg4.1 and Δatg4.2 null mutants were readily obtained by two successive rounds of targeting their endogenous loci, multiple attempts to generate a line that lacked both ATG4.1 and ATG4.2 genes were unsuccessful. Single allele deletions of the second ATG4 gene in both Δatg4.1 and Δatg4.2 lines were successful (to give Δatg4.2::atg4.1$^{-/-}$ and Δatg4.1/atg4.2$^{-/-}$, respectively), but the second round of allele replacement using these lines resulted in trisomic parasite lines in which both selectable markers had integrated into the appropriate endogenous locus but there appeared an additional copy of the endogenous gene. As both of these loci had been successfully targeted in generating the Δatg4.1 and Δatg4.2 mutants, the results of these genetic manipulation attempts suggest strongly that the parasite requires at least one copy of either ATG4.1 or ATG4.2.

$L. major$ Mutants Lacking ATG4s Form GFP-ATG8-containing Puncta—Δatg4.1 and Δatg4.2 both grew indistinguishably from WT as promastigotes in vitro. GFP-ATG8 has been validated as a molecular marker for autophagy in *Leishmania* and has been used to show that autophagy increases during differentiation between life cycle stages with the abundance of autophagosomes in promastigotes being greatest during metacyclogenesis (33, 41). Thus we used this marker protein with the...
mutant lines to investigate the involvement of the two ATG4s in autophagy. The lines were transfected with pN-GFP-ATG8 and the occurrence of GFP-labeled puncta was monitored (Fig. 2A). The \( \Delta \text{atg}4.1 \) lines (designated \( \Delta \text{atg}4.1[pN-\text{ATG8}] \) and \( \Delta \text{atg}4.1::\text{ATG4.1}[pN-\text{ATG8}] \)) at the mid-logarithmic phase of growth produced one to two GFP-labeled autophagosomes per cell, which was similar to WT cells. In contrast \( \Delta \text{atg}4.2[pN-\text{ATG8}] \) had multiple puncta. With \( \Delta \text{atg}4.1 \), the proportion of cells bearing the GFP-labeled structures peaked at day 7 (when metacyclogenesis was maximal), similarly to the situation with WT promastigotes although the number of cells with puncta was lower (Fig. 2B). The proportion of cells bearing GFP-labeled structures returned to WT levels in \( \Delta \text{atg}4.1::\text{ATG4.1}[pN-\text{ATG8}] \) in both cases, the proportion of cells with GFP-labeled puncta subsequently declined to low levels as stationary phase was reached and metacyclogenesis ended.

We have shown previously that autophagy in \( L. \) major is induced by starvation (33), so we quantified the number of autophagosomes in the mutants after starvation (Fig. 2D). The proportion of cells with GFP-ATG8 puncta increased dramatically in WT, \( \Delta \text{atg}4.1 \), \( \Delta \text{atg}4.1::\text{ATG4.1} \), and \( \Delta \text{atg}4.2::\text{ATG4.2} \) promastigotes. In contrast, the proportion of \( \Delta \text{atg}4.2 \) promastigotes with GFP-ATG8 puncta was initially relatively high and this only increased a little under starvation conditions.
The Requirement for the Different ATG4s in Autophagy Involving ATG8A—Previously, we showed that the preferred substrate for ATG4.2 was ATG8A (32). To investigate further the relative roles of ATG4.1 and ATG4.2 in the biogenesis of ATG8A-containing puncta, ∆atg4.1[pN-ATG8A] and ∆atg4.2[pN-ATG8A] lines were generated, incubated in starvation medium, and monitored for the formation of ATG8A-containing puncta (Fig. 2E). ∆atg4.1[pN-ATG8A] produced multiple GFP-ATG8A-labeled structures per cell, similar to WT promastigotes. In contrast ∆atg4.2[pN-ATG8A] produced no such puncta, as described previously (32). The proportion of promastigotes with GFP-ATG8A puncta under starvation conditions was ~90% in WT, ∆atg4.1, ∆atg4.1::ATG4.1, and ∆atg4.2::ATG4.2 (Fig. 2F), a great increase from 2 to 5% in non-starved cells. No puncta were detected in ∆atg4.2 promastigotes, strongly supporting the hypothesis that ATG8A-dependent puncta formation is totally dependent upon ATG4.2.

Metacyclogenesis and Infectivity of the Δatg4 Mutants—We reported previously that ∆atg4.2 promastigotes are defective in metacyclogenesis (33), thus we investigated whether ∆atg4.1 has a similar defect. ∆atg4.1 promastigotes differ from ∆atg4.2 promastigotes in that they apparently differentiated fully to metacyclic promastigotes, as judged by the lectin peanut agglutinin assay (Fig. 3A) and the presence of the metacyclic-specific markers, SHERP and HASPB (Fig. 3B); the process seemed delayed and there was a significant difference in the number of metacyclic promastigotes at day 7 between the WT parasites...
Leishmania ATG4 Cysteine Peptidases

FIGURE 3. Metacyclogenesis and infectivity of L. major ATG4 null mutants. A, the percentage of metacyclic promastigotes in stationary phase populations of promastigotes was determined by the peanut lectin agglutination method. Values shown are the mean ± S.D. from three independent experiments. *, data for ATG4.1 differed significantly from those for WT promastigotes at day 7 (p < 0.01). B, extracts of 10^7 stationary phase promastigotes at day 7 (D7) or day 10 (D10) were assessed for metacyclic-specific proteins by Western blot analysis with anti-SHERP and anti-HASPB antibody. Cysteine synthase (CS) served as a loading control. C and D, mouse peritoneal macrophages were infected in vitro with stationary phase promastigotes or amastigotes isolated from lesions in BALB/c mice. Cells were assessed at 1 and 5 days post-infection for the percentage of infected macrophages. Data are mean ± S.D. and are representative of three independent experiments. *, infection level of ATG4 null mutant lines differed significantly from those of WT parasites (p < 0.05). E–H, BALB/c mice were infected with 2 × 10^7 promastigotes or amastigotes and footpad lesion size was monitored weekly. Data are mean ± S.D. of five mice. *, infection level of ATG4 null mutant lines differed significantly from those of WT or the re-expressing line (p < 0.05).

and ATG4.1 (p < 0.05), but by day 10 there was no difference (Fig. 3A).

We compared the two mutants for infectivity to macrophages in vitro and mice. Stationary phase promastigotes of both ATG4.1 and ATG4.2 infected macrophages in vitro in similar numbers to WT parasites but each of them survived poorly over 5 days with many macrophages clearing the infection (Fig. 3C). In contrast, amastigotes of ATG4.1, purified from mice, were as infective as WT amastigotes and proliferated equally well (Fig. 3D). Amastigotes of ATG4.2 also infected macrophages well but survived poorly over 5 days (Fig. 3D, right panel).

Analysis of the infectivity of ATG4.1 and ATG4.2 lines to BALB/c mice using promastigotes and amastigotes yielded results that correlated well with those from the in vitro analysis using macrophages (Fig. 3, E–H). Lesions resulting from inoculation of stationary phase ATG4.1 and ATG4.2 promastigotes were slower growing than those of WT parasites, this was reversed by re-expression of the genes (Figs. 3, E and G). In contrast, inoculation of ATG4.1 amastigotes generated lesions similar to those of WT amastigotes (Fig. 3F), whereas inoculation of ATG4.2 amastigotes resulted in slower growing lesions (Fig. 3H). Lesion development with amastigotes of ATG4.2 was at an intermediate level between WT and ATG4.2 (Fig. 3H). These findings suggest that both ATG4s play some role in facilitating infection of mice by promastigotes, and that ATG4.2 is also important in the survival and multiplication in mice of inoculated amastigotes, whereas ATG4.1 is not.

Morphology of ATG4.2 in Mice—The finding that inoculation of ATG4.2 amastigotes into BALB/c resulted in slower growing lesions than WT parasites prompted us to investigate whether the lesions were equivalent in terms of parasite numbers per volume. The results showed that the number of amastigotes contained within lesions of comparable width was significantly lower for ATG4.2 than with WT and ATG4.1 (p < 0.05; Fig. 4A). Scanning electron microscopy (SEM) analysis of ATG4.2 purified from lesions showed that they had a longer body length on average than WT amastigotes (p < 0.05; Fig. 4B and C), a difference largely reversed by re-expression of ATG4.2 in ATG4.1 (p < 0.05). Lesion development with amastigotes of ATG4.2 was not significantly different in size from WT amastigotes (p > 0.05; data not shown).

Transformation of the parasites extracted from lesions in mice back to promastigotes was assessed by inoculating them into 5 ml of medium at the cell density of 2 × 10^7 ml^-1. Under these conditions, ATG4.2 parasites differentiated into promastigotes more quickly than the WT and ATG4.1 parasites (Fig. 4D).

Use of the ATG4 Mutants to Study the Relationship between ROS Levels and Autophagy in Leishmania—The relationship between ROS levels and induction of autophagy known for mammalian cells prompted us to study whether there is a correlation between ROS levels and the abundance of autophagosomes in Leishmania. The approaches we adopted were measuring ROS levels in the mutants, correlating these with autophagosome generation, and measuring the sensitivity of the autophagy-compromised mutants to oxidative stress. We also modulated the ROS levels in promastigotes through the addition of the antioxidant NAC (known to scavenge H_2O_2, hydroxyl radical, and superoxide within cells and to help maintain a reduced intracellular environment (42)), and monitored how this affected autophagy.

NAC added to the starvation buffer inhibited the biogenesis of GFP-ATG8-containing puncta in all cell lines except ATG4.2 promastigotes (Fig. 2D). The proportion of ATG4.2 promastigotes with GFP-ATG8-containing autophagosomes was not statistically different whether or not NAC was added (Fig. 2D). These data show an apparent correlation between increased...
ROS levels and number of GFP-ATG8 puncta in starved promastigotes (except for ∆atg4.2) and are consistent with autophagy being induced by ROS as reported for other cells (24, 25, 43). However, the accumulation of GFP-ATG8 containing puncta in ∆atg4.2 even when ROS levels had been diminished using NAC confirm that this mutant has dysfunctional autophagic machinery.

The addition of NAC also abrogated the formation of the GFP-ATG8A structures (Fig. 2F). These data suggest that ATG8A-dependent puncta formation is similar to autophagy involving ATG8 in being induced by ROS.

Increased ROS Levels and Oxidatively Damaged Proteins in ∆atg4—To elucidate the parts played by each L. major ATG4 in any autophagic response to counter ROS, we measured the levels of ROS in normal and starved promastigotes of the various lines using 2,7-dichlorofluorescin (H2DCF-DA)(24, 25, 44, 45). This revealed that ROS levels in ∆atg4.2 were 2-fold higher ($p < 0.01$) than in WT promastigotes at early log phase (day 2), whereas those in ∆atg4.1 were similar to WT (Fig. 5A, compare with Fig. 2B). ROS levels were increased 40-fold in WT promastigotes during a 2-h starvation, a similar scale of increase also occurred in the two null mutant lines (Fig. 5A). Addition of NAC at 2.5 mM to the starvation medium suppressed ROS levels at 2 h very greatly in promastigotes of all lines except ∆atg4.2 (Fig. 5A). More NAC (5 mM) was required to produce an equivalent reduction of ROS in ∆atg4.2 (Fig. 5A).

ROS levels during in vitro growth of promastigotes were also assessed to determine whether there was any apparent relationship between these and the occurrence of autophagy. The levels in WT promastigotes increased as early log phase progressed to mid log phase and early stationary phase but then declined at stationary phase (Fig. 5B, time points equivalent to days 2, 4, 6, and 9 in Fig. 2). Thus the period at which ROS levels were highest coincided with the time when autophagy was also elevated. The same profile occurred with ∆atg4.1 and ∆atg4.1::ATG4.1. However, the situation was different in the
Leishmania ATG4 Cysteine Peptidases

**Figure 5.** Increased ROS levels and oxidatively damaged proteins in Δatg4.2 promastigotes. **A,** ROS levels, measured using 50 mM H2DCF-DA, in early log promastigotes incubated for 2 h at 27 °C in PBS with or without NAC. Note the discontinuity in the ordinate axis. Data are mean \( \pm \) S.D. from independent experiments. *, ROS levels in extracts of promastigotes at the growth phases detailed in Table I, with or without NAC. **B,** ROS levels in WT promastigotes at early log (EL), mid log (ML), early stationary (ES), and stationary (S) phases of growth (days 2, 4, 6, and 9, respectively). **C,** Fluorescence of MitoTracker Green staining, which is used to assess total mitochondrial mass, upon the mitochondrion of the parasite. MitoTracker Green (40 kDa) was detected in extracts of WT promastigotes and also that more than three-quarters of the mitochondrial network was lost or fragmented mitochondrial morphology. However, there was a similar overall intensity of the fluorescence in all the cell lines, consistent with there being little difference in mitochondrial mass and this remained the same in the presence of 5 mM NAC (data not shown). A much lower fluorescence intensity was observed with MitoTracker Red (which assesses mitochondrial membrane potential) in Δatg4.2 compared with WT promastigotes (Fig. 6B, closed bars; \( p < 0.05 \)), suggesting that mitochondrial function was impaired in this null mutant. No such difference was noted for the other mutants (Fig. 6B, open bars). Addition of NAC to normal medium greatly increased viability of the WT itself was reduced to \( \sim 80 \% \) compared with that when in growth medium.

The sensitivity of the various lines to exogenous hydrogen peroxide was also assessed. The results show that Δatg4.2 promastigotes are more susceptible than the other lines, with an IC\(_{50}\) of 0.21 ± 0.02 mM compared with IC\(_{50}\) values of around 0.5 mM for the other lines. These findings suggest that deletion of ATG4.1 does not interfere with the ability of the promastigote to withstand these stresses, whereas removal of ATG4.2 does have a detrimental effect.

**Δatg4.2 Promastigotes Have a Dysfunctional Mitochondrion—** There is increasing evidence that general autophagy is required for the maintenance of mitochondrial homeostasis, and we have recently shown this to be so for *Leishmania* by generating mutants lacking ATG5 (31). Thus we investigated whether removal of the individual ATG4s from *L. major* had an impact upon the mitochondrion of the parasite. MitoTracker Green staining, which is used to assess total mitochondrial mass, showed that Δatg4.1 promastigotes were similar to WT promastigotes at early log phase, increased as log phase progressed, and was maintained at this high level during stationary phase. This phenotype was restored to WT levels in the Δatg4.2::ATG4.2 re-expressor.

One consequence of elevated ROS in a cell is the unwanted oxidation of proteins, which can be removed via autophagy. Protein carbonyl content has been shown to be a biomarker for protein oxidation (46), thus we investigated the presence of carbonylated proteins in parasite extracts by derivatization with 2,4-dinitrophenylhydrazine. The stable dinitrophenylhydrazone product was assessed by Western blot analysis. Just a single protein of \( \sim 60 \) kDa was detected in extracts of WT promastigotes, as previously reported (47, 48), and only in those of promastigotes at early stationary phase of growth when ROS levels are highest (Fig. 5C). However, the same analysis with Δatg4.2 yielded the equivalent protein also in extracts of stationary phase promastigotes (Fig. 5C). Thus for this mutant there is an apparent correlation between elevated ROS levels, protein carbonylation, and restricted autophagic flux.

**Δatg4.2 Promastigotes Are Less Able to Withstand Starvation and Oxidative Stresses—** Autophagy generally has a role in protecting against starvation and other stresses, thus we tested the hypothesis that deletion of ATG4 would hinder autophagic flux and make promastigotes of *L. major* more susceptible to stresses. Δatg4.2 promastigotes were indeed less able to withstand starvation in PBS, with viability, as assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, after 2 h starvation being 42% less than that of WT, Δatg4.1, Δatg4.1::ATG4.1, and Δatg4.2::ATG4.2 promastigotes under the same conditions (Fig. 5D). Viability of the WT itself was reduced to \( \sim 80 \% \) compared with that when in growth medium.

The sensitivity of the various lines to exogenous hydrogen peroxide was also assessed. The results show that Δatg4.2 promastigotes are more susceptible than the other lines, with an IC\(_{50}\) of 0.21 ± 0.02 mM compared with IC\(_{50}\) values of around 0.5 mM for the other lines. These findings suggest that deletion of ATG4.1 does not interfere with the ability of the promastigote to withstand these stresses, whereas removal of ATG4.2 does have a detrimental effect.

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still disrupted in 15 ± 1% of promastigotes at this time point (Fig. 6E). Prolonged incubation in normal medium containing NAC for up to 48 h maintained ROS at relatively low levels (Fig. 6C, open bars) and the mitochondrial membrane potential close to but significantly lower than that of WT promastigotes (Fig. 6, compare D with B) and there was also a significant decrease in the proportion of cells with a defective mitochondrion (to 6 ± 1%, Fig. 6E). These results suggest that the lack of ATG4.2 and consequent impaired autophagic flux is responsible for the increased levels of ROS and that these lead to impaired mitochondrial homeostasis and function.

**DISCUSSION**

This study aimed to elucidate the roles of the two ATG4 enzymes in *L. major* by generating gene deletion mutants. Both individual null mutants were generated successfully using promastigotes and their phenotypes were subsequently analyzed. The main defects observed for Δatg4.1 were the fewer promastigotes that contained GFP-ATG8-containing autophagosomes (Fig. 2B) and poorer ability of promastigotes to successfully infect macrophages *in vitro* (Fig. 3, C and D) and in mice (Fig. 3, E-H), even though they appeared to undergo metacyclogenesis *in vitro* similarly to, although slightly slower than, WT parasites (Fig. 3, A and B). The capacity of this mutant to produce autophagosomes, albeit less abundantly than the WT parasites, implies some redundancy in function between the two ATG4s. We have previously shown that although ATG4.2 has higher activity toward the ATG8A group of paralogues, it can hydrolyze ATG8 *in vitro* after prolonged incubation (32) and this is supported by the observation in this study of GFP-ATG8-containing puncta in mutants lacking ATG4.1. However, puncta containing ATG8B and ATG8C, also preferred substrates for ATG4.1 (32), were not formed in Δatg4.1 (data not shown). Thus the viability of Δatg4.1 shows that these proteins and the puncta that they form do not have an essential role in the parasite. The reduced ability of promastigotes of Δatg4.1 to infect macrophages and mice may reflect a reduction in flux through the autophagic pathway and so, perhaps, a reduced capacity for differentiation between life cycle forms. Thus these

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**FIGURE 6. Evidence for a dysfunctional mitochondrion in Δatg4.2 but not Δatg4.1.** A, the types of mitochondrial morphology observed by fluorescence microscopy in promastigotes stained with MitoTracker Green. Scale bar, 10 μm. B, the fluorescent intensity from MitoTracker Red, at 0.1 μM, of 2 × 10⁶ promastigotes in normal medium after 4 h incubation at 26 °C with and without NAC. Data are mean ± S.D. from three independent experiments. * and ** denote statistically significant differences between, respectively, WT and Δatg4.2 and Δatg4.2 with and without NAC. C–E, effect of addition of NAC to Δatg4.2 promastigotes at 2 × 10⁶ ml⁻¹ in normal medium over 48 h. C, ROS levels measured using 50 mM H₂DCF-DA. D, fluorescent intensity from MTR. E, the proportion of Δatg4.2 promastigotes with an abnormal mitochondrial morphology.

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*Leishmania ATG4 Cysteine Peptidases*

*FEBRUARY 1, 2013• VOLUME 288 • NUMBER 5 JOURNAL OF BIOLOGICAL CHEMISTRY*
data are consistent with the contributions of ATG4.1 to autophagy being significant but not essential for *Leishmania* development.

The deletion of ATG4.2 had more profound effects upon the parasite. The lack of ATG4.2 markedly reduces the ability of the parasite, whether as promastigotes and amastigotes, to infect and survive in both macrophages in *vitro* (Fig. 3, C and D) and also mice (Fig. 3, E–H). As Δatg4.2 had a significantly higher amount of lipidated ATG8 (Fig. 2C) and a higher number of GFP-ATG8-containing puncta at stationary phase of growth (Fig. 2B), we conclude that the lower virulence of this mutant is due primarily to a block in the delivery of autophagosomes to the lysosomal network, probably due to poor cleavage of ATG8 from the mature autophagosomes, and thus a reduction in flux through the ATG8-dependent autophagic pathway. To further characterize the identity of the higher number of GFP-ATG8-containing puncta in Δatg4.2 at stationary phase of growth, we transfected the line with mC-ATG5, which also labels autophagosomes (31). These analyses showed that most of the puncta were labeled with GFP-ATG8 only, very rarely was labeling observed with mC-ATG5 (data not shown). As ATG5 is released from nascent autophagosomes before they mature, this is entirely consistent with our idea that the additional puncta in Δatg4.2 are mature autophagosomes. Unfortunately there are no other markers for autophagosomes currently available for use with *Leishmania*. However, electron microscopic analyses also revealed that structures typical of autophagosomes (as exemplified in Ref. 41) were more abundant in Δatg4.2 than in Δatg4.1 or WT parasites (data not shown).

The significance of the deconjugation of ATG8-PE by ATG4 at the early stage of the autophagic cycle (31), thus the data for Δatg4.2 are consistent with this. There is again with autophagic flux being reduced in this mutant and a role for autophagy in dealing with ROS stress, hypotheses that can be tested in future studies.

There was also a prominent depolarization of the mitochondrial membrane potential and dysfunctional mitochondria (52, 53). Our investigation of the relationship between ROS and autophagy in *Leishmania* has shown that ROS levels do vary as *Leishmania* promastigotes grow *in vitro* with, interestingly, higher levels occurring during the phase when metacyclogenesis occurs and autophagy is elevated (Fig. 5B). Δatg4.2 had higher levels of ROS than WT throughout this growth, with notably higher levels at stationary phase when the numbers of autophagosomes are also markedly elevated. This is consistent again with autophagic flux being reduced in this mutant and a role for autophagy in dealing with ROS stress, hypotheses that can be tested in future studies.

Autophagy plays a part in protecting against ROS-mediated damage of proteins and organelles in eukaryotic cells (52), and its absence results in elevated ROS levels and consequently accumulation of oxidized proteins, a lower mitochondrial membrane potential and dysfunctional mitochondria (52, 53). Moreover, autophagy can be induced by elevated ROS (24–26). Our investigation of the relationship between ROS and autophagy in *Leishmania* has shown that ROS levels do vary as *Leishmania* promastigotes grow *in vitro* with, interestingly, higher levels occurring during the phase when metacyclogenesis occurs and autophagy is elevated (Fig. 5B). Δatg4.2 had higher levels of ROS than WT throughout this growth, with notably higher levels at stationary phase when the numbers of autophagosomes are also markedly elevated. This is consistent again with autophagic flux being reduced in this mutant and a role for autophagy in dealing with ROS stress, hypotheses that can be tested in future studies.

Our experiments using the antioxidant NAC in parasites starved for 2 h showed that this agent resulted in lower ROS levels in all lines (Fig. 5A) and also to decreased production of autophagosomes containing ATG8 and ATG8A (Fig. 2) and restoration of mitochondrial integrity (Fig. 6). This is consistent with ROS being an important regulator of autophagy in *Leishmania* and also supports ROS as a mediator of mitochondrial damage, although direct evidence needs to be obtained in future studies to confirm this.

In human cells, ROS modulates the autophagic pathway during amino acid starvation (25). This appears to occur through ROS inhibiting the deconjugation activity of ATG4, causing ATG8-PE to accumulate at the pre-autophagosomal mem-
branes. This fosters autophagosomal biogenesis, and so autophagy to remove more rapidly oxidized proteins and damaged organelles (24, 45). This mechanism cannot operate in aged organelles (24, 45). This mechanism cannot operate in autophagy to remove more rapidly oxidized proteins and damaged membranes. This fosters autophagosome biogenesis, and so a precise mechanism is likely to differ from that in yeast. For a gene encoding ATG21 and so if such inhibition occurs the organism has a regulatory mechanism in which ATG4 is similarly differs from mammalian cells in this way and this organism has a regulatory mechanism in which ATG4 is blocked from ATG8-PE in nascent autophagosomes by the ATG12-ATG5 complex, ATG18 and ATG21 (8). It remains to be determined whether such a blockage functions in Leishmania but we have shown that the parasite does have the ATG5-ATG12 conjugation pathway (31) and a gene encoding ATG18 is present in the L. major genome (www.genedb.org, LmjF29.1575). However, there is not currently strong evidence for a gene encoding ATG21 and so if such inhibition occurs the precise mechanism is likely to differ from that in yeast.

Our results overall show that the ATG4s of L. major are different, there is some redundancy in function between them especially in autophagosome biogenesis, but that ATG4.2 would appear to be more important than ATG4.1 for the later stages of the autophagic pathway when ATG8 is cleaved from the autophagosomes. Neither is individually essential, and the finding that cells lacking both ATG4.1 and ATG4.2 could not be obtained, even though both loci were demonstrated to be targetable by the gene deletion constructs, suggests that ATG4 performs essential roles in Leishmania. The activity of the enzymes toward not only ATG8 but also ATG8A, both of which function in autophagy in some conditions, makes autophagy in Leishmania an interesting system to investigate in terms of the roles of the individual ATG4 proteins.

The results presented also support the idea that autophagy is important for differentiation of the parasite, and especially in enabling promastigotes to infect mammals. Importantly, however, ATG4.2 appears to have a constitutive role in the growth of the parasite in mammals. This is an important finding that opens up many new avenues for further investigation on the roles of autophagy in the Leishmania-mammal interaction.

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