Expansion of the target of rapamycin (TOR) kinase family and function in *Leishmania* shows that *TOR3* is required for acidocalcisome biogenesis and animal infectivity

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Target of rapamycin (TOR) kinases are key regulators of cell growth, proliferation, and structure in eukaryotes, processes that are highly coordinated during the infectious cycle of eukaryotic pathogens. Database mining revealed three TOR kinases in the trypanosomatid parasite *Leishmania major*, as defined by homology to the phosphoinositide 3-kinase–related kinase (PIKK) family and a signature conserved FKBP12/rapamycin-binding domain. Consistent with the essential roles of TOR complexes in other organisms, we were unable to generate null *TOR1* or *TOR2* mutants in cultured *L. major* promastigotes. In contrast, *tor3*$^{-}$ null mutants were readily obtained; while exhibiting somewhat slower growth, *tor3*$^{-}$ maintained normal morphology, rapamycin sensitivity, and differentiation into the animal-infective metacyclic stage. Significantly, *tor3*$^{-}$ mutants were unable to survive or replicate in macrophages in vitro, or to induce pathology or establish infections in mice in vivo. The loss of virulence was associated with a defect in acidocalcisome formation, as this unique organelle was grossly altered in *tor3*- mutants and no longer accumulated polyphosphates. Correspondingly, *tor3*- mutants showed defects in osmoregulation and were sensitive to starvation for glucose but not amino acids, glucose being a limiting nutrient in the parasitophorous vacuole. Thus, in *Leishmania*, the TOR kinase family has expanded to encompass a unique role in AC function and biology, one that is essential for parasite survival in the mammalian infective stage. Given their important roles in cell survival and virulence, inhibition of TOR kinase function in trypanosomatids offers an attractive target for chemotherapy.

chemotherapy | protozoan parasite | Trypanosomatidae | virulence | Kinetoplastida

Leishmaniasis is typically classified as a "neglected tropical disease," with 12 million persons infected worldwide. This disease is caused by trypanosomatid protozoan parasites of the genus *Leishmania*, which are transmitted by Phlebotomine sand flies and cause a range of symptoms from self-healing skin lesions to lethal visceral ulcers. In the past 15 y, coinfections with the HIV have increased the burden of leishmaniasis in public health, especially in sub-Saharan Africa, where access to treatment still remains a challenge (1).

The infective cycle of *Leishmania* includes a series of developmental steps, as promastigotes within the midgut of the sand fly and as amastigotes within the phagolysosome of the mammalian host cell. In these different environments, the parasites must adapt to variable conditions of temperature, pH and nutrient availability (2–4). Understanding the molecular mechanisms used by *Leishmania* parasites to survive under these harsh conditions is fundamental to our understanding of the disease, and ultimately for its control.

Target of rapamycin (TOR) kinases are key master regulators in eukaryotes, linking environmental conditions such as nutrient availability and stimuli to protein synthesis and to the cell cycle machinery to coordinate cell growth and replication. In humans, abnormal regulation of the mammalian TOR (mTOR) pathway has been associated with several diseases, such as cancer and type 2 diabetes (5). Initially, TORs were identified by mutations that conferred resistance to a potent antifungal metabolite called rapamycin (6), which acts against many TOR kinases through formation of an inhibitory complex with the peptidyl-prolyl *cis*/*trans* isomerase FKBP12 (7). TOR kinases belong to the family of phosphoinositide 3-kinase–related kinases (PIKKs), a protein family playing roles in cellular response to various types of stresses (8, 9). PIKKs possess a C-terminal domain similar to the catalytic domain of PI3 and PI4 lipid kinases, but acting as Ser/Thr kinases. PIKKs exhibit several other conserved domains such as FAT and FATC domains flanking the kinase domain, and HEAT domains in the N terminus, which participate in protein–protein interactions (8, 9). One defining feature of TORs is the FKBP12/rapamycin-binding (FRB) domain, originally identified as the binding site for FKBP12/rapamycin complexes but since shown to mediate important TOR functions (10–12).

Whereas most organisms encode one or two TORs that act within complexes known as TORCs, we found three TOR kinase genes bearing the signature FRB domain in *Leishmania major* and other trypanosomatids. To investigate their role, we attempted to produce null mutants for each. Although *TOR1* and *TOR2* appear to be essential, consistent with their essential function in organisms including trypanosomes (13), we were able to generate null mutants of *TOR3*. Notably, *tor3*- grew and differentiated normally in culture but was highly attenuated in macrophage and mouse infection. These phenotypes were associated with a specific defect in the synthesis of acidocalcisomes (ACs), a unique organelle of protozoans playing an important role in energy metabolism.

Results

Identification of TOR Kinases in *L. major*. Database mining of the genome of *L. major* for TOR-related genes revealed four candidates (Fig. S1 and Table S1), showing similarity (43–50%) spanning ~1,200 aa encompassing the C terminus, and including characteristic PIKK family domains, including HEAT, FAT, PI-kinase domain, and FATC. Only three bore the TOR signature FRB domain (14–16), and consistent with the nomenclature established recently in *T. brucei* (13), we termed them *TOR1* (LmjF36.6320), *TOR2* (LmjF34.4530), and *TOR3* (LmjF34.3940; this is termed TOR-like1 in trypanosomes; Fig. 1*A*). Only *TOR3* showed a PDZ motif, a protein-interaction domain often found in signaling complexes located at cell membranes (17) (Fig. 1*A*). The catalytic kinase domain is well conserved in all three *L. major* TOR kinases, including the residue corresponding to Asp^{2338} in mTOR (Fig. S2) required for kinase activity (18).

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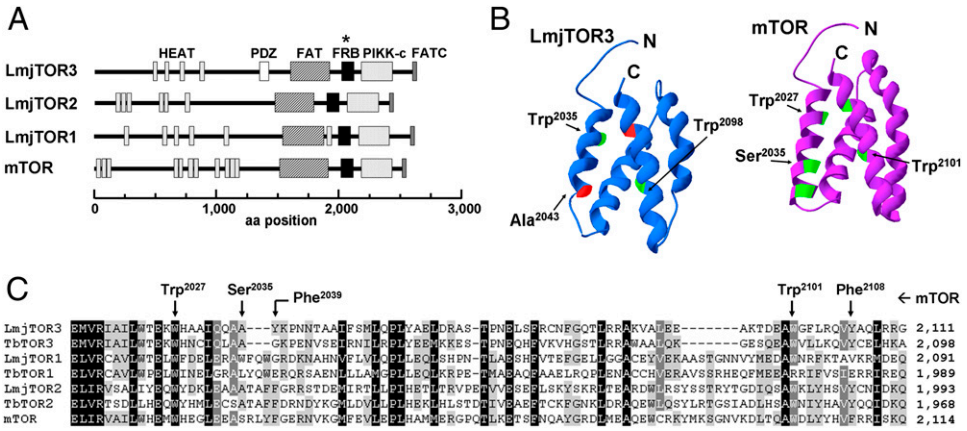

Fig. 1. Three conserved TOR kinases in the genome of *L. major*. (*A*) Domain organization of *L. major* TOR kinases. Star highlights the signature TOR kinase FRB domains. (*B*) Modeling of the *L. major* TOR3 FRB domain, developed using the Phyre remote homology modeling server (19). The structure of the modeled LmjTOR3 FRB is shown at the left, compared with the crystal structure of human mTOR at the right (20). Critical residues for rapamycin binding in mTOR FRB and conserved/mutated amino acids in the corresponding positions of LmjTOR3 FRB are highlighted. (*C*) Multiple sequence alignment of the FRB domain of the three TOR kinases of *L. major*, *T. brucei,* and human mTOR. Darker shading corresponds to a higher degree of conservation. Arrows point to conserved residues for rapamycin binding in mTOR (NP_004949).

The powerful protein structural homology and modeling system PHYRE (19) was used to identify and model the FRB domains of *L. major* TOR1/2/3 and TbTOR2 and TbTOR-like 1 (Fig. 1 *B* and *C* and Table S1), against the structure of the mTOR FRB domain (10, 20). The Trp residues corresponding to mTOR Trp^{2027} and Trp^{2101} required for kinase activation and FKBP12/rapamycin binding are conserved (Fig. 1 *B* and *C*) (6, 20–23). However, Trp^{2101} was replaced by Arg in TbTOR1, consistent with the finding that rapamcyin–FKBP complexes target TORC2 rather than TORC1 in the trypanosome bloodstream forms (13). All trypanosomatid TORs showed substitutions at residues corresponding to mTOR Ser^{2035} (Ala in TOR2s and TOR3/TbTOR-like 1, Trp in *L. major* and Leu in trypanosome TOR1s), which disrupt FKBP12/rapamycin binding to mTOR (21). Correspondingly, *L. major* promastigotes and the insect and bloodstream forms of trypanosomes are relatively insensitive to rapamycin (13, 24).

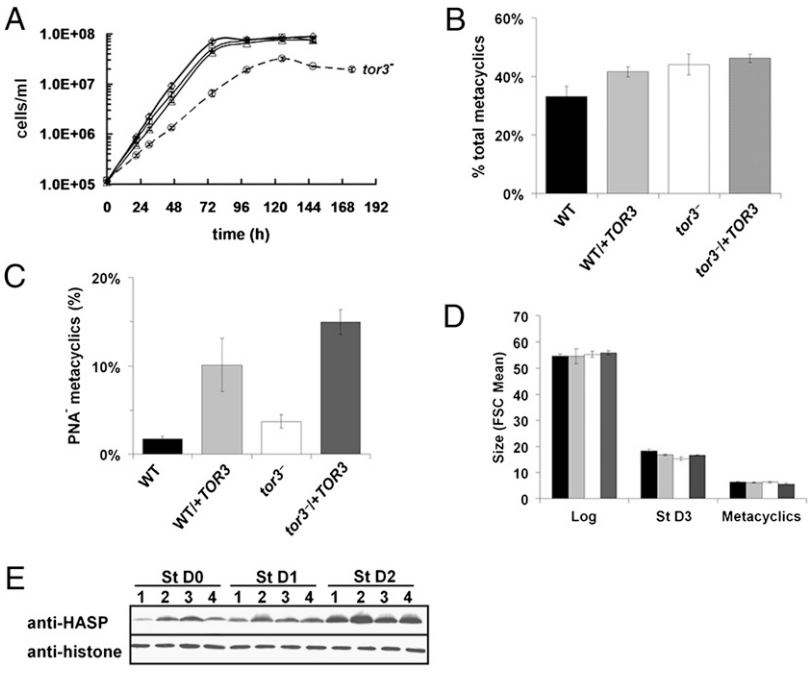

Fig. 2. *L. major tor3*$^{-}$ promastigotes have a slower-growth phenotype but do not show defective metacyclogenesis. (*A*) Growth of *L. major* promastigotes in vitro. WT (◇), WT/+*MycTOR3* (□), *tor3*$^{-}$ (○), and *tor3*$^{-}$/+*MycTOR3* (Δ). Error bars show SE mean for two independent experiments. (*B*) Percentage of metacyclics in the total cell population on the second day of stationary phase (St D2) as determined by flow cytometry and gating for the smaller metacyclic vs. promastigote populations. Because *tor3*$^{-}$ grows more slowly and reaches stationary phase with a 1-d delay, St D2 occurs at 147 h for *tor3*$^{-}$ and 125 h for the other lines. Bars in Figs. 2–4 are shaded: WT, black; WT/+*TOR3*, gray; *tor3*$^{-}$, white; and *tor3*$^{-}$/+*TOR3*, darker gray. (*C*) Percentage of PNA^{-} metacyclics, measured on St D2 as described above. (*D*) Size of promastigotes as measured by flow cytometry using the forward scatter (FSC) during logarithmic growth (Log), stationary phase (St D3), or of purified PNA^{-} metacyclics (isolated on St D3). (*E*) Total cell lysates of promastigotes harvested upon entry or after 1 or 2 d in stationary phase (St D0,1,2) were analyzed by Western blotting with antisera to the metacyclic promastigote protein HASPB (28). Lanes are as follows: 1, WT; 2; WT/+*TOR3*; 3, *tor3*$^{-}$; and 4, *tor3*$^{-}$/+*TOR3*. Antihistone H2A antibodies were used as loading control.

L. major TOR1 and _TOR2_ Are Likely Essential Genes, but _tor3_$^-$ Null Mutants Are Viable as Promastigotes. The *Leishmania* genome is predominantly diploid, and two rounds of gene replacement are required to obtain null mutants (25). For both *TOR1* and *TOR2*, heterozygotes were readily obtained, but we were unable to generate homozygous knockouts of either gene despite multiple well-controlled attempts (Fig. S3 *A* and *B*). These results suggest that *L. major TOR1* and *TOR2* may be essential.

In contrast, homozygous *tor3*$^-$ null mutants were readily obtained by two successive rounds of targeting (Figs. S3*C* and S4 *A* and *B*). An episomal expression construct bearing an N-terminal Myc tag (pXNG4SAT-MycTOR3) was introduced into both *tor3*$^-$ and WT (termed *tor3*$^-$/+*TOR3* and WT/+*TOR3*, respectively). Western blotting with anti-Myc antibodies revealed a band of molecular weight greater than 250 KDa in both lines, in agreement with the 295-KDa expected size of Myc-TOR3 (Fig. S4*C*).

TOR3 Affects Growth Rate but Not Cell Size of _L. major_ Promastigotes. The *tor3*$^-$ mutant grew slower in culture, with a 10- to 11-h doubling time vs. 8 h for WT, reaching a stationary phase density of ~25% that of WT (Fig. 2*A*). Both *tor3*$^-$/+*TOR3* and WT/+*TOR3* grew normally (Fig. 2*A*), showing that slower growth arose from loss of *TOR3*. All lines showed the same morphology and size in both log and stationary growth phases [55 ± 0.7 and 16.6 ± 1.2 arbitrary units (AU)], as assessed by forward light scatter by flow cytometry (26) (Fig. 2*D*).

Upon entry into stationary phase during in vitro culture, a significant fraction of *Leishmania* parasites differentiate into infective metacyclic forms, accompanied by changes in cell morphology, size, gene, and surface protein expression (2, 27, 28). First, the metacyclic marker protein HASPB was expressed similarly in WT, *tor3*$^-$, *tor3*$^-$/+*TOR3,* and WT/+ *TOR3* (Fig. 2*E*). Second, the percentage of "small" metacyclics was assessed by flow cytometry and size gating; the percentage of small cells was similar, with the *tor3*-, *tor3*$^-$/+ *TOR3,* and WT/+*TOR3* lines somewhat increased relative to WT (33 ± 4% vs. 42–46 ± 1–3%; Fig. 2*B*). Third, a subset of metacyclic parasites fail to bind the lectin peanut agglutinin (PNA), which can be exploited to purify these parasites (27, 29). PNA$^-$ metacyclics again showed a similar size (6.4 ± 0.3 AU; Fig. 2*D*). Finally, *tor3*$^-$ parasites showed no reduction in levels of PNA$^-$ metacyclic formation (WT, 1.7 ± 0.3%, vs. *tor3*$^-$, 3.6 ± 0.8% ; Fig. 2*C*). The *tor3*$^-$/+ *TOR3* and WT/+*TOR3* cell lines showed an increase in the percentage of PNA$^-$ metacyclics (10.1 ± 3.0% and 14.9 ± 1.4%, respectively). This may reflect the properties of the episomal expression vector, which typically results in overexpression (30). These data establish that *tor3*$^-$ mutants are not defective in metacyclogenesis and are not altered in size.

Infectivity of _L. major tor3_$^-$ to Mice Is Highly Attenuated. In footpad inoculations of susceptible BALB/c mice, metacyclic or stationary phase WT parasites induced progressive lesion pathology in a dose-dependent manner. Although heterozygous TOR3 replacements resembled WT, several independent *tor3*$^-$ lines failed to induce any lesion pathology even when inoculated at high levels (10^7 stationary cells) more than 1 y postinfection. In contrast, 10 independent *tor3*$^-$/+*TOR3* lines showed pathology similar to that of WT. We followed lesion pathology in Balb/C mice quantitatively after inoculation with lower numbers of parasites (10^6 stationary cells or 10^5 metacyclics); again, *tor3*$^-$ mutants failed to show pathology, which was restored by complementation with episomal expression of *TOR3* (Fig. 3*A* and Fig. S5). As often seen in *Leishmania* virulence studies, complementation with the episomal vector was imperfect, with a 2- to 3-wk delay in lesion formation relative to WT. Nonetheless, the substantive restoration compared with the complete attenuation of *tor3*$^-$ argues that the effect on virulence is specific.

Several studies were undertaken to establish whether parasites persisted in the absence of pathology. After inoculation of 10^6 stationary phase cells, parasites were recovered from only 1/16 total *tor3*$^-$ infected mice after 19–25 wk (580 ± 170 parasites). In a second experiment, mice were examined at 21 d postinfection; WT and WT/+ *TOR3* infections yielded comparable numbers of parasites (~10^7 /foot), whereas no *tor3*$^-$ parasites were recovered (Fig. 3*B*). Partial rescue was again seen with *tor3*$^{-/+}$ *TOR3* (~10^5/foot). Increased survival of *tor3*$^-$ was seen with higher inocula. Although lesions were never observed with 10^7 stationary phase parasites (16 mice), 7/8 mice showed small lesions (0.08–0. 6 mm) when inoculated with 10^8 parasites. These were accompanied by a correspondingly low level of parasitemia (0–1,000 with the 10^7 inocula, to 10^4–10^5 with the 10^8; Fig. S6). Thus, under some conditions, limited persistence of *tor3*$^-$ occurred. However, preliminary studies of recovered persistent parasites did not reveal any evidence of secondary changes leading to elevated virulence.

tor3$^-$ Is Unable to Survive in Macrophages. Although *tor3*$^-$ parasites showed normal invasion of peritoneal exudate macrophages, these parasites were rapidly eliminated afterward, decreasing 80% within the first 2 d and nearly eliminated after 5 d (Fig. 3*C*). Restoration of *TOR3* in turn rescued parasite survival, albeit to levels lower than seen in WT. Interestingly, the WT/+*TOR3* line also showed somewhat reduced macrophage survival (Fig. 3*C*), again pointing to effects associated with TOR3 overexpression from episomal vectors. These data argue that *TOR3* is required for establishment and survival of *L. major* in macrophage infections.

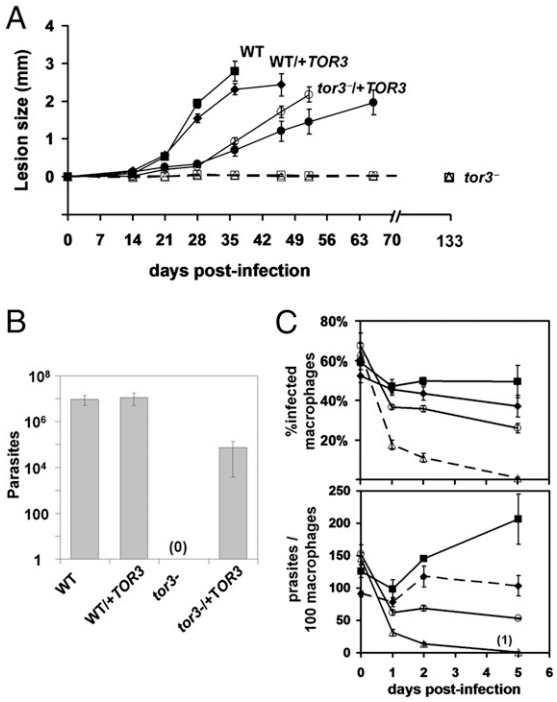

Fig. 3. *L. major tor3*$^-$ mutant has highly attenuated virulence during the amastigote stage. (*A*) Increase in footpad thickness after inoculation of 10^5 purified PNA$^-$ metacyclics into Balb/C mice; WT (■), WT/+*TOR3* (◆), two clonal lines of *tor3*$^-$ (□, △) and *tor3*$^-$/+*TOR3* (○,●). SEs are shown (n = 4). (*B*) Parasitemia was measured by limiting dilution assay at day 21 postinfection in an experiment similar to that shown in (*A*), except that the inoculums was 10^6 stationary phase cells/foot. SEs are shown (n = 4). (*C*) Macrophage infections. WT (■), WT/+*TOR3* (◆), *tor3*$^-$ (△) and *tor3*$^-$/+*TOR3* (○). Three independent experiments were performed, with triplicate samples within each; data show results of one representative experiment. Bars indicate SEs for triplicate wells.

***tor3*⁻ Shows an ACs Defect.** Although *tor3*⁻ promastigotes appeared morphologically normal, transmission EM showed alterations in ACs, which appeared to be 2-fold smaller (0.6 vs. 0.3 μM; Fig. 4*A* and Fig. S7). ACs are acidic organelles that store calcium and other divalent cations, as well as pyrophosphate and polyphosphates (Poly-P) (31). ACs were visualized by reactivity with antisera to the marker *T. brucei* VP1, a vacuolar proton pyrophosphatase (32). This confirmed the smaller AC size and increased numbers, which suggested some AC fragmentation in *tor3*⁻ (Fig. 4*B*). Both phenotypes were reversed by restoration of *TOR3* (Fig. 4 *A* and *B*). DAPI staining was used to visualize Poly-P accumulation, which upon binding exhibits strong fluorescence emission at 530 nm (33, 34). DAPI staining was greatly reduced in the *tor3*⁻ mutant, which again was reversed upon restoration of *TOR3* (Fig. 4*C*). These data establish that *tor3*⁻ is defective in ACs formation and Poly-P accumulation therein.

TOR3 Is Involved in Environmental Stress Sensing. Both TOR kinases and ACs have been implicated in sensing environmental signals including nutrients and osmotic stress (2–4, 35). *tor3*⁻ was exposed to hypo-osmotic stress, and changes in cell volume were monitored by light scattering, in which a decrease in absorbance corresponds to an increase in volume (36). As expected, in WT and *tor3*⁻/+*TOR3* promastigotes, the volume quickly increased, peaking after 1 min and recovering after 3 min (Fig. 5*A*). However, *tor3*⁻ parasites showed a more pronounced increase in volume and required 5 min to stabilize.

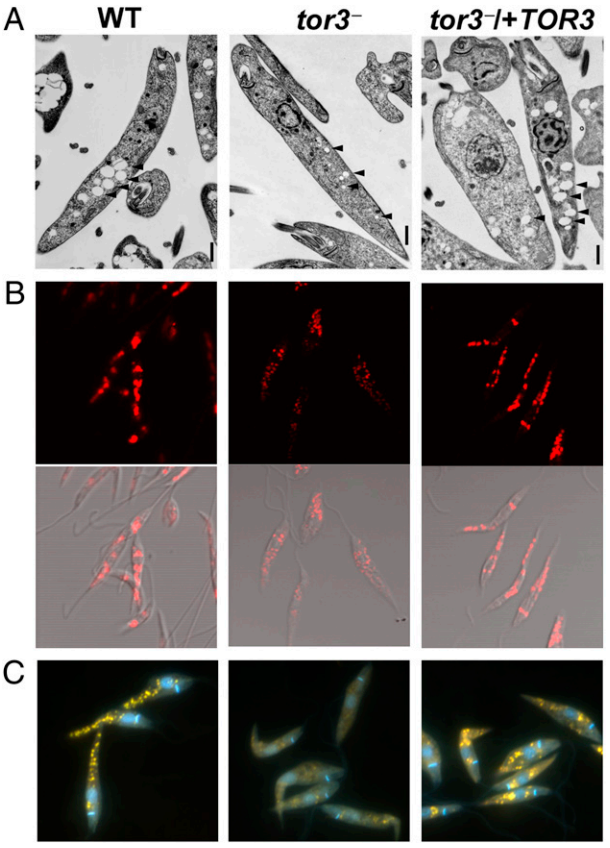

Fig. 4. *L. major tor3*⁻ promastigotes show alterations in ACs structure and polyphosphate content. (*A*) Transmission EM reveal much smaller ACs (arrows) in *tor3*⁻ compared with WT or *tor3*⁻/+*TOR3* parasites. (Scale bars, 1 μm.) (*B*) Immunofluorescence with anti-TbVP1 antibody. (*Upper*) Results. (*Lower*) superimposition of results on DIC image. (*C*) Polyphosphate staining. DAPI staining of paraformaldehyde-fixed promastigotes in late stationary phase to detect polyphosphates (yellow fluoresecence) in the ACs.

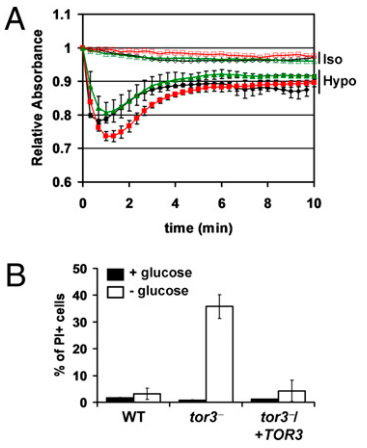

Fig. 5. Lack of TOR3 in *L. major* promastigotes show alterations in response to hyposmotic stress and elevated sensitivity to glucose starvation. Promastigotes were subjected to isotonic (open symbols) or hypo-osmotic (filled symbols) conditions, and the absorbance monitored over time as described in *Materials and Methods*. (*A*) WT (◇, ◆), *tor3*⁻ (□, ■), and *tor3*⁻/+*TOR3* (△, ▲). Volume recovery was measured by light scattering. Result shown is for one of two similar experiments, each containing duplicate samples. (*B*) Glucose tolerance. Parasites were grown in RPMI media with glucose (closed bars) or without glucose (open bars). After 4 d, viability was assessed by uptake of propidium iodide using flow cytometry (PI^+ indicates dead cells). Error bars are SDs of two independent experiments.

Next, *tor3*⁻ promastigotes were tested for their sensitivity to starvation for serum, amino acids, or glucose. In contrast to TOR mutants in other species, *tor3*- showed no alterations in sensitivity to amino acids and serum starvation. However, *tor3*⁻ promastigotes were highly sensitive to glucose starvation; after 4 d in culture medium lacking glucose ≈35% of the mutant parasites were dead, in contrast to only 2% for WT or *tor3*⁻/+*TOR3*⁻ cells (Fig. 5*B*). Thus the absence of *TOR3* confers sensitivity to glucose but not amino acid or serum starvation.

Discussion

TOR kinases are evolutionarily conserved in eukaryotes, where they play a major role in sensing and integrating nutrient/energy status and mitogens in the environment to promote coordinated cell growth and replication (reviewed in refs. 15, 37, 38). Although most organisms encode only 1 or 2 TOR kinases, in *L. major* we identified at least three members of the TOR kinase family. *L. major* TOR1 and TOR2 are orthologous to trypanosome TbTOR1 and TbTOR2, which carry out typical TOR kinase functions such as growth control, size, and macroautophagy (TbTOR1), or cell polarization, endocytosis, and cytokinesis (TbTOR2) (13, 39, 40). Inducible RNAi knockdowns suggested Tb*TOR1* and TbTOR2 were essential, and similarly we were unable to generate null mutants in *TOR1* or *TOR2* in *Leishmania major*. In the future, it may be possible to carry out further tests of *TOR1* and *TOR2* function in *Leishmania*, by generating hypomorphic or conditional mutations using a unique protein-based regulatory system (24), or by exploiting the possibility of RNAi technology in the distantly related species *L. braziliensis* (41).

We identified a third TOR kinase*TOR3,* based on the clear presence of an FRB domain (Fig. 1). This important TOR domain was originally identified as the binding site of FKBP12/rapamycin complexes to some but not all TORs, and since has been shown to mediate important TOR functions independent of this, including binding to phosphatidic acid (10–12). Trypanosomatid TORs show some diversity in FRB structure (Fig. 1*C*) and function relative to other species, as evidenced by the binding of TbFKB12 to TbTOR2 but not TbTOR1 or TOR-like 1 (13).

TOR3: A TOR Kinase with "Nonclassical" Function in *Leishmania* Virulence. Notably, the role of TOR3 in *L. major* is distinct from that of other TORs. *tor3*$^-$ Null mutants were readily obtained, and in culture appeared remarkably normal, growing only somewhat more slowly than WT, and retaining the ability to differentiate to the infective metacyclic stage in vitro, without affecting cell size or shape (Fig. 2). However, the virulence of the *tor3*$^-$ mutant in susceptible Balb/C mice model was dramatically attenuated, and *tor3*$^-$ was unable to survive or replicate in macrophages cultured in vitro (Fig. 3). Only after inoculation of massive numbers of parasites could persistence or occasional lesion pathology occur. Importantly, all phenotypes could be reversed, fully or partially, upon restoration of *TOR3* expression.

TOR3 and AC Function. Unexpectedly, *tor3-* showed strong alterations in ACs. ACs are ancient organelles, typically associated with storage of cations such as calcium and zinc, and polyphosphates of various chain lengths (31). ACs play important roles in calcium homeostasis, intracellular pH, osmoregulation, and phosphate metabolism, and through the high-energy phosphoanhydride Poly-P bonds, as a crucial source of energy (31, 42, 43). EM and immunofluorescence microscopy showed that the *tor3*$^-$ mutant had altered ACs, with a fragmented appearance consisting of numerous smaller vesicles, most clearly evident when visualized with a specific AC membrane marker (Fig. 4 and Fig. S7). *tor3*$^-$ ACs showed functional alterations as well, as evident by a severe defect in Poly-P accumulation (Fig. 4C). Consistent with a role for ACs and Poly-P in adaptation to extreme conditions and energy metabolism, *tor3*$^-$ was highly sensitive to glucose starvation (Fig. 5B), and showed defects in responding to osmotic shock (Fig. 5A). Thus, in every parameter studied, the AC in the *tor3*$^-$ was both structurally and functionally compromised. Again emphasizing its functional divergence from other TORs, *tor3*$^-$ was insensitive to serum or amino acid starvation.

How might TOR3 control AC function? TOR kinases generally occur in complexes (37), and TOR3 may also occur in a complex, which in trypanosomes differs from those formed by both TOR1 and TOR2 (13). Although the basis of TOR kinase specificity is not well understood, cellular localization and membrane localization are likely to be important, and TOR3 bears a PDZ domain often implicated in protein–protein interactions and membrane targeting (13, 44–46) (Fig. 1*A*). The strong effects of *TOR3* on AC function raised the possibility that TOR3 may be localized to this organelle, and preliminary immunoblotting data suggest the MycTOR3 protein localizes to particulate fractions. However we have been unable to visualize TOR3 by fluorescence or immune EM microscopy, despite the use of a variety of methods and antisera. The identification of the components of a putative TOR3 complex as well as its specific substrates are the obvious next steps toward a deeper understanding of the pathways involved in the control of *Leishmania* virulence by this protein kinase.

ACs and/or Poly-Ps have been shown to play important roles in adaptation to changing environmental conditions, including starvation and osmotic stress, which are encountered by *Leishmania* and other pathogens in the interactions with mammalian hosts (reviewed in refs. 31, 42, 43).

Alterations in both ACs and poly-P levels have been associated with reduced virulence in trypanosomatids (32–34, 43, 47–52), and the importance of AC during the infectious cycle is also supported by quantitative studies of AC abundance, which show their numbers to increase 3- to 6-fold in amastigotes (53). The inability of *L. major tor3*$^-$ parasites to survive in the macrophages or mice may reflect their increased sensitivity to glucose starvation (Fig. 5*B*), as the phagolysosome is thought to be a compartment poor in hexoses (54, 55), and deregulation of other important AC functions in the *tor3*$^-$ mutants may similarly account for their poor survival in the mammalian host. The similarity in the phenotypes seen in studies of AC protein mutants with those of *tor3*$^-$ leads us to propose that the *tor3*$^-$ phenotype(s) may arise solely from their affect on AC. In the future, studies of *tor3*$^-$ will help to elucidate the dynamics involved in the origin and maintenance of the structure of the ACs.

Altogether, our data show that *L. major* TOR3 has remarkably little phenotype during growth in vitro, but is required for survival and disease pathology in the mammalian host. In addition to shedding light on the role of the AC and TOR kinases in virulence, these studies have several practical implications. First, the limited persistence of *tor3*$^-$ under some conditions suggests some potential in live vaccination studies. Second, TOR3 inhibition may serve as an attractive target for chemotherapy. Recent work has led to the development of drugs that target the mTOR pathway (56–59). The strong divergence of trypanosomatid TORs generally and the function of TOR3s specifically suggest that there may similarly be an opportunity for the design of selective antiparasite TOR agents in the future.

Materials and Methods

***Leishmania* Culture and Isolation of Metacyclics.** *L. major* Friedlin clone V1 (MHOM/IL/81/Friedlin) and LV39 clone 5 (Rho/SU/59/P) were grown in M199-based medium supplemented with 10% FBS, 62.5 μM adenine, and 2 μg/mL biopterin (60). Cell density was determined by using a model Z1 Coulter counter (logarithmic phase) or hemocytometer (stationary phase). Metacyclics were isolated by negative selection with PNA (29) or by Ficoll gradient centrifugation (27).

In starvation studies, promastigotes were grown in M199 medium until mid log phase (~8×10^6 cells/mL), washed in incomplete RPMI lacking serum, amino acids, and glucose, and then split into flasks with different RPMI compositions. Complete RPMI (Gibco-BRL) medium was supplemented with 30 mM Hepes, pH 7.4, 62.5 μM adenine, 2 μg/mL biopterin, 5 μg/mL hemin, and 1% (vol/vol) heat-inactivated FCS (61).

Cell volume was measured by forward-scatter using a FACSCalibur cytometer (Beckton-Dickinson), and analyszed used CellQuest software. Samples were incubated with propidium iodide (0.5μg/mL) in M199 for 5 min at room temperature to assess viability. Methodology for assays of AC-related phenotype in the *tor3*$^-$ mutant, such as EM, immunofluorescence, and DAPI staining are detailed in *SI Text*. Regulatory volume decrease response was measured by light scatter (36). Briefly, 10^8 late log-phase promastigotes were washed twice in isotonic Iso-Cl buffer (20 mM Hepes, pH 7.4, 11 mM glucose, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 137 mM NaCl, 4 mM KCl, 1.5 mM K_2HPO_4, and 8.5 mM Na_2HPO_4), and resuspended in 550 μL Iso-Cl, after which 250-μL aliquots were added to cuvettes containing an equal volumes of Iso-Cl or water (hypotonic stress). Absorbance at 550 nm was measured using a Beckman DU640 spectrophotometer at 10-s intervals for 10 min.

Targeted Gene Replacement of *L. major* TORs. TOR kinase domains were identified in searches of the Pfam database (62), and the FRB domain model was viewed using the Swiss-PdbViewer (63). We used gene replacement by homologous recombination to generate knockout lines of each of the *L. major TOR* kinases. Details on the replacement targeting cassettes, primers, and procedures used are provided in *SI Text* and Table S2.

Infectivity Studies. *L. major* FV1 promastigotes (stationary phase or purified metacyclics) were injected s.c. into the left hind footpads of 6- to 8-wk-old female BALB/C mice (Charles River Laboratories). Unless otherwise stated, parasites were harvest after 3 d in the stationary phase. Lesion sizes were measured using by Vernier calipers, and parasite numbers were enumerated by limiting dilution assay (64). In vitro infection of peritoneal macrophages was performed as described previously (65, 66), using metacyclics isolated from a Ficoll density gradient (27).

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