Centrin Gene Disruption Impairs Stage-specific Basal Body Duplication and Cell Cycle Progression in *Leishmania*

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Centrin is a calcium-binding cytoskeletal protein involved in the duplication of centrosomes in higher eukaryotes. To explore the role of centrin in the protozoan parasite *Leishmania*, we created *Leishmania* deficient in the centrin gene (*LdCEN*). Remarkably, centrin null mutants (*LdCEN<sup>−/−</sup>*) showed selective growth arrest as axenic amastigotes but not as promastigotes. Flow cytometry analysis confirmed that the mutant axenic amastigotes have a cell cycle arrest at the G<sub>2</sub>/M stage. The axenic amastigotes also showed failure of basal body duplication and failure of cytokinesis resulting in multinucleated “large” cells. Increased terminal deoxy uridine triphosphate nick end labeling positivity was observed in centrin mutant axenic amastigotes compared with wild type cells, suggesting the activation of a programmed cell death pathway. Growth of *LdCEN<sup>−/−</sup>* amastigotes in infected macrophages *in vitro* was inhibited and also resulted in large multinucleated parasites. Normal basal body duplication and cell division in the *LdCEN* knockout promastigote is unique and surprising. Further, this is the first report where disruption of a centrin gene displays stage-specific/cell type-specific failure in cell division in a eukaryote. The centrin null mutant defective in amastigote growth could be useful as a vaccine candidate against leishmaniasis.

The centrins are basal body/centriole-associated calcium-binding proteins. Centrin has been characterized in eukaryotes from unicellular organisms such as *Chlamydomonas* (1, 2) to humans (3–5). Fibers composed of centrin and its binding protein Sf1p act as contractile or elastic connections between centrioles/basal bodies and other elements of the centrosome to mediate dynamic changes in its overall structure (6, 7). Conditional mutations in yeast centrin (CDC31) result in failure of duplication of the spiral pole body (yeast centrosome) and arrest in mitosis (8, 9). Silencing the centrin gene in the water fern *Marsilea vestita* effectively inhibits the formation of motile cells (10). Similarly, silencing of human centrin 2 (*HcCEN2*) in HeLa cells impaired cilium duplication and resulted in the failure of cytokinesis (4). A centrin-deficient mutant of *Chlamydomonas* was nonflagellate because of defects in the flagellar root system (2). Abnormal centrosomes were noticed in human tumor cells because of the presence of excess levels of many centrosome proteins including centrin (11). We recently cloned several centrin genes<sup>1</sup> and characterized one such a gene, which is involved in the growth of the protozoan parasite, *Leishmania donovani* (12).

Leishmaniasis currently threatens 1.5–2.0 million people annually with an estimated death toll of 50,000 persons/year in 88 countries around the world (13). Most of the fatalities are due to visceral leishmaniasis. Treatment for this disease involves chemotherapy using antimony-based drugs, which is less effective in immunocompromised individuals and usually associated with drug resistance (14). No effective vaccine has been developed successfully for this disease (13). The parasite *L. donovani*, a causative agent of human visceral leishmaniasis, has a digenic lifestyle with one form called the promastigote form that resides extracellularly in the mid-gut of the sand-fly vector and with another form called the amastigote form that multiplies intracellularly in vertebrate macrophages. These two forms have been adapted to grow under *in vitro* conditions (14, 15). To understand the molecular mechanisms of growth control, we cloned and characterized the gene encoding a basal body-associated protein centrin (*LdCen*) (12). *L. donovani* centrin protein (*LdCenp*) sequence similarity and immunoreactivity confirmed that *Leishmania* centrin (*LdCEN*) is a homolog of human centrin 2 (12). The high level expression of LdCenp during log phase growth and reduction of growth by overexpression of an N-terminally deleted centrin in both promastigote and amastigote forms indicate that centrin has an important role in *Leishmania* growth (12). To study the mechanism of centrin function in this lower eukaryote, we targeted the *LdCEN* gene for a null mutation.

As a method to study gene function, targeted deletion has been successful only for few *Leishmania* genes. Dihydrofolate reductase-thymidylate synthase and *Leishmanolysin* genes were deleted in *Leishmania major* (16–19), and genes involved in Lipoxygenase were deleted in *Leishmania mexicana* and *L. major* (20–22). These mutant parasites clearly demonstrated the physiological role of the genes that were deleted. Unlike many other eukaryotes, *Leishmania* is diploid throughout its life cycle. Hence it is necessary to delete both the alleles to generate a null mutant for a specific gene (16). In this report we describe a stepwise disruption of the two alleles of *LdCEN*, using genes resistant to antibiotics hygromycin B and G418 (Geneticin) and

<sup>1</sup>A. Selvapandiyan, unpublished data.
characterization of the *LdCEN* null mutant parasites for their growth both as extracellular promastigotes and axenic amastigotes as well as intracellular amastigotes inside macrophages. The purpose of the study is to establish a correlation between centrin expression and parasite growth and to characterize the *LdCEN* null mutants as a first step to evaluate their potential as a live attenuated vaccine in the future.

**EXPERIMENTAL PROCEDURES**

In *Vitro Culture of Parasites and Molecular Biology*—The parasite culture procedure and the routine molecular biology practices were as previously described (12).

**Construction of DNA for Targeted Gene Deletion**—For *L. donovani* centrin, nearly two-thirds (1,323 bp) of the gene including the translation initiation site ATG was added upstream of the selectable marker genes, nucleotides forward (P7) and reverse (P8) primers. To ensure translation of these genes during PCR amplification (see Fig. 1, M seq), P5 (5′-GG-GTG ACC ACC CAA AAG AAA TTTG-3′) contains a SalI restriction site (bold). The PCR product was cloned directly at the SpeI cloning site of *pCR4-TOPO* (Invitrogen). The recombinant plasmid having the insert with the SpeI end toward the Sp6 promoter was cut with SpeI and KpnI and used in the next step.

The 5′-UTR of *LdCEN* was amplified by PCR using a cosmid clone containing the *LdCEN* gene as template (12) and the following forward (P1) and reverse (P2) primers. P1 (5′-GG-GTG ACC CTA ATT ATG GCC ACG CAT G-3′) contains a BamH1 restriction site (bold). P2 (5′-GGG TCG ACC CAA AAA TAA TTTG-3′) contains a Sall restriction site (bold). The PCR product was cloned directly at the Sall cloning site of *pCR4-TOPO* (Invitrogen). The recombinant plasmid having the insert with the Sall end toward the Sp6 promoter was cut with SpeI and KpnI and used in the next step.

The 3′-UTR of *LdCEN* was PCR-amplified using the cosmid clone mentioned above as template and the following forward (P3) and reverse (P4) primers. P3 (5′-GAC-TAG TAC TGC TGG TGG AGC ACC-3′) contains a SpeI restriction site (bold). P4 (5′-GGG TAC TTA TTC ACC GCC TCG G-3′) contains a KpnI restriction site (bold). The PCR product was restricted with Sall and KpnI and ligated at the same sites of the cut plasmid product of step A. The resulting recombinant plasmid was cut with Sall and SpeI and used in the following step.

The selectable marker sequence *hyg* was amplified using plasmid *pMSE-HYG* (16) as template and forward (P5) and reverse (P6) primers. The *neo* gene was amplified using plasmid *pKSN/neo* (23) as template and forward (P7) and reverse (P8) primers. To ensure translation of these marker genes, nucleotides (16 to +12 of the 3′-nucleotidase/nuclelease gene including the translation initiation site ATG was added upstream of the *hyg* and *neo* genes during PCR amplification (see Fig. 1, M seq). P5 (5′-GG-TGC GAC GCT AGC GCA GAC CAT GCT CAA GGT gaa cct gaa ctc-3′) has a SpeI restriction site (bold) and the 3′-nucleotidase/nuclelease sequence as mentioned earlier (single underlined), and the first 18 nucleotides of *hyg* orf (lowercase). P6 (5′-GG-GGA CTA GTC TAT TCC GTC CCA GCT G-3′) has a Sall restriction site (bold) followed by 3′ end sequence of *hyg* gene. P7 (5′-GGG TAC TTA TTC ACC GCC TCG G-3′) is a similar primer to primer P5 except the 3′ end has the first 18 nucleotides of *neo* orf (lowercase). P8 (5′-GAC-TAG TCA GAA GAA CTC GGA CGA GCT AAG-3′) has a Sall restriction site (bold) followed by the 3′ end sequence of the *neo* gene. Both the PCR-amplified *hyg* and *neo* fragments were cut with Sall and SpeI restriction enzymes, and the fragments were ligated at the cut plasmid obtained in the second step. The authenticity of each of the constructs and the fidelity of the PCR-amplified fragments were verified by nucleotide sequencing. The resulting *hyg* and *neo* recombinant plasmids were cut with BamH1 and KpnI, and the fragments of either *hyg* or *neo* were ligated with the UTR sequences of *LdCEN* (constructs 1 and 2, respectively; see Fig. 1) and individually used for transfection to disrupt *LdCEN* in *L. donovani*.

**Transfection and Selection of *LdCEN* Null Mutants**—Mid-log phase promastigotes (2–4 × 10⁷ cells/ml) were harvested by centrifugation at 3,000 × g for 10 min at 4° C. The cell pellets were washed in ice-cold PBS and electroporated with the DNA constructs using conditions as described previously (12). For clonal selection, the transfected promastigotes were incubated overnight in 5 ml of *L. donovani* growth medium. The cultures were centrifuged as above, and the pellet was resuspended in 200 µl of the promastigote growth medium, syringe-disrupted with a 28-gauge needle, and plated on 1% agar plate containing the same growth medium supplemented with 4 µg/ml Bioterin (Geneticin; Invitrogen) and the antibiotics G418 (Geneticin; Invitrogen) and hygromycin B (Sigma) at appropriate concentrations.

Transfection with construct 1 was performed as described above and finally plated on culture plate containing 80 µg/ml of hygromycin B. Clones isolated from the plates were subsequently expanded in liquid medium containing 40 µg/ml of hygromycin B. Genomic DNA isolated from these cloned parasites was used in Southern blot analyses (12) to confirm the loss of one allele of centrin. Such a cell line in which one allele of the centrin gene was substituted by the *hyg* gene was subjected to the next round of transfection using Construct 2, and clones were selected on culture containing 80 µg/ml of hygromycin B and 40 µg/ml of G418 antibiotics. The clones were analyzed for the complete knockout of the centrin gene and the presence of both *hyg* and neo in Southern and Western blot analyses as described previously (12). The parasites were grown both as promastigotes and axenic amastigotes in the absence of antibiotics except the single knockout (+/−) that was grown in the presence of hygromycin B 40 µg/ml to avoid elimination of the *hyg* gene by the duplication of the single centrin allele.

Light and Fluorescence Microscopy—For DAPI fluorescence the parasites grown at different time points were washed once with 1× PBS, treated with trypan blue, air-dried on the microscopic slide, and mounted in Vectashield containing DAPI (Vector Laboratories, Inc.). DAPI stains both nucleus and kinetoplast DNA. The cells were examined for fluorescence under the ECLIPSE TE2000-U microscope (Nikon Corporation, Tokyo, Japan) with epifluorescence, and the images were captured with a digital camera (Hamamatsu C4742–95, Hamamatsu Photonics K.K.) and processed with Open Lab software (Improvision, Inc.). For indirect immunofluorescence studies, 200 µl of cultured cells were harvested and mounted on slides using a Cytoplasm (Shandon, Pittsburgh, PA). The preparation was fixed in −20° C methanol for 10 min, blocked (10% goat serum, 5% glycerol, 0.1% Nonidet P-40 in PBS) for 30 min, and incubated with 1:2,000 anti-centrin (20H5 ascites fluid) for 30 min. The preparation was then washed thoroughly with PBS, incubated in fluorescein isothiocyanate-conjugated secondary antibody for 30 min, washed again, and stained for DNA using DAPI. The cells were subsequently analyzed by fluorescence microscopy as above.

**Electron Microscopy—**Parasites from 48-h cultures were processed and examined by electron microscopy as described previously (24).

Flow Cytometry—Flow cytometry was carried out as described (12). Briefly, axenic amastigotes from 24- and 48-h cultures were collected, fixed in 70% ethanol, stained with 50 µg/ml propidium iodide (PI from Sigma) in PBS, and analyzed. For each sample 20,000 fluorescent events were measured. To analyze the plasma membrane integrity, promastigotes were inoculated at 1 × 10⁶ cells/ml into axenic amastigote culture medium, and samples of axenic amastigote cells were collected at various times after inoculation. Fluorescence of the cells treated with propidium iodide (PI) was measured following our published protocols (24). The data are expressed as percentages of PI positive parasites. For each sample 10,000 fluorescent events were measured. All the above analyses were carried out using FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and CELLQuest software, and the data were analyzed using the ModFit LT Software (Verity Software House, Inc., Topsham, ME).

**TUNEL Assay—**Mid-log phase promastigotes were inoculated into axenic amastigote culture medium and allowed to grow for different periods of time. At each time point the cells were harvested by centrifugation and prepared for TUNEL staining according to the manufacturer’s protocol (Roche Applied Science). A negative control was included in each staining wherein only the labeling solution was added. The samples were stained with Vectastain ABC-Elite Kit (Vector Laboratories, Inc.). The specimens were then observed and counted using a fluorescence microscope.

In *Vitro Macrophage Infections*—Human elutriated monocytes were resuspended at 1.8 × 10⁵ cells/ml in RPMI medium containing macrophage colony-stimulating factor (25), plated in 0.5 ml on eight chamber slides (Lab-Tek tissue culture slides, Miles Laboratories), and incubated for 8 days for differentiation into macrophages. The differentiated macrophages were infected with stationary phase cultures of promastigotes (10:1, parasite to macrophage ratio) as described previously (25). After incubation for 5 h at 37° C in 5% CO₂, the free extracellular parasites were removed by repeated washings in RPMI, and the cultures were incubated in macrophage medium for 18, 24, 48, 72, and 240 h post-infection, the culture medium was removed from a sample of the culture slides, and the slides were air-dried, fixed by immersion in absolute methanol for 5 min at room temperature, and stained using Diff-Quick Stain set (Baxter Healthcare Corporation, 2 The abbreviations used are: orf, open reading frame; UTR, untranslated regions; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2′-phenylindole-dihydrochloride; PI, propidium iodide; TUNEL, terminal deoxy uridine triphosphate nick end labeling.
For each culture, a minimum of 300 macrophages were counted. The results are expressed either as percentages of macrophages that were infected by *Leishmania* or as the mean number of parasites/infected macrophage.

Restoration of Centrin in *LdCEN*/*H11002* Parasites—To restore centrin in the *LdCEN*/*H11002* parasites, *LdCEN* orf was first PCR-amplified using a *LdCEN* containing plasmid (12) as template and the following forward (P9) and reverse (P10) primers. P9 (5'-GGG ATC CAT GGC TGC GCT GAC GGA T-3') contains a BamHI restriction site (bold). P10 (5'-GGG ATC CTT ACG CTT AGT CCG GCA GGT GGT Act ttc cac gca tgt gca g-3') contains sequentially a BamHI restriction site (bold), a sequence for an hemagglutinin tag (underlined), and 18 nucleotides of 3' end of the *LdCEN* gene sequence (lowercase letters). The amplified product was initially cloned at the T/A cloning site of *pCRII-TOPO*.

**Fig. 1.** Schematic diagram showing design and use of constructs for *LdCEN* gene disruption in the *L. donovani* genome. *Constructs 1 and 2* show *hyg* and *neo* genes, respectively, flanked on the 5' and 3' sides with *LdCEN* 5'-UTR and 3'-UTR, respectively. The lengths of the antibiotic-resistant genes and the *LdCEN* untranslated regions have been described under “Experimental Procedures.” M-seq, 14 nucleotides upstream, the initiation codon and three codons downstream in the *L. donovani* 3'-nucleotidase/nuclease gene (39) fused in frame to the antibiotic resistance coding sequences to improve translation. BamHI, KpnI, Sall, and SpeI are the restriction sites.

**Fig. 2.** A, schematic drawing of the centrin locus in *L. donovani* with SalI restriction sites. Disrupting the centrin gene with either *hyg* or *neo* gene generates an additional SalI site at the 5' end of the selectable marker genes. *B*, Southern blot analysis of the genomic DNA of *Leishmania* wild type (+/+), *LdCEN* single (+/-), and double (-/-) allele disrupted parasites with *hyg*, *neo*, and *LdCEN* genes as 32P-labeled probes. The genomic DNA from the parasites was cut with the restriction enzyme SalI and used in the analysis. *C*, Western blot analysis of the lysates of +/+ , +/− , and −/− parasites using anti-LdCen antibody (12). Lower panel, after the protein transfer the membrane was stained with Ponceau S to show equivalence of protein loading. *D*, the effect of *LdCEN* disruption on the *in vitro* growth of *Leishmania* promastigotes and axenic amastigotes of wild type (+/+), centrin single allele disrupted (+/−), and centrin null mutant (−/−). The cells were grown in the absence of antibiotics. Initial cell density in the culture was 0.1 × 10⁷ cells/ml. The data represent the means ± S.D. of four independent experiments. No significant difference in growth was observed between +/− and +/+ parasites.
FIG. 3. A, cell cycle analysis by flow cytometry of *L. donovani* LdCEN*+/+* (+/+) and LdCEN*−/−* (−/−) parasites. Promastigotes inoculated at 1 × 10⁶ cells/ml in axenic amastigote medium were allowed to grow 24 or 48 h. The resulting axenic amastigotes were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in each cell cycle stage, G1, S, or G2/M, is plotted for the 24-h time point (left panel) and 48-h time point (right panel). The solid bars indicate the wild type cells (+/+), and the open bars indicate the centrin-deficient (−/−) cells. The data represent the means ± S.D. of three independent experiments. *, *p* < 0.008; **, *p* < 0.0005 Student’s t test.

B, axenic amastigotes of +/+ and −/− from the 48-h culture were stained with DAPI and viewed under a fluorescent microscope. Right panel, the pale blue spots are kinetoplasts, and the larger dark blue regions are nuclei. Left panel, phase contrast image of the same field shown in the right panel. Scale bar, 5 μm.

c, transmission electron micrograph of *Leishmania* wild type (+/+), and mutant (−/−) axenic amastigotes after 48 h in culture. The kinetoplast (K) and nucleus (N) are indicated. Scale bar, 2 μm. D, Quantitative analysis of the number of DAPI-stained nuclei in the LdCEN*−/−* axenic amastigotes with increasing time in culture. Cells, with one, two, or more than four nuclei, were counted and individually plotted as percentages of cells showing each number of multi-nucleated cell. The results were obtained from at least 150 viable cells (cells not staining to trypan blue) observed in each case. The data represent the means ± S.D. of three independent experiments. E, indirect immunofluorescence analysis of centrin distribution in wild type promastigote (Pro, panel a) and axenic amastigote (Am, panel b), and LdCEN*−/−* promastigote (panel c) and axenic amastigote (panel d) cells. DAPI stains DNA of nucleus and kinetoplast (blue) and monoclonal antibody to *Chlamydomonas* centrin (20H5) stains a centrin other than LdCenp (green). Centrin staining at the flagellar base and along the axoneme is evident in promastigote cells (single cell enlarged and shown in the inset in panel a) of both wild type (panel a) and LdCEN*−/−* (panel c) and at the flagellar basal apparatus in wild type amastigote cells (panel b). LdCEN*−/−* amastigotes are largely devoid of 20H5 staining (panel d). Scale bar, 5 μm. F, electron
cloning vector. The fidelity of the cloned sequence was verified by nucleotide sequencing. The BamHI insert was subsequently cloned at the same site of pXG-PHLEO vector (26), and the recombinant plasmid, pXG-PHLEO-LdCEN, was transfected into the LdCEN−/− promastigotes as described previously (12). Transfected promastigotes were selected with minimal doses of phleomycin (Sigma) (10 μg/ml). In our experience, expression of protein through an episome is enhanced by increasing the concentration of selection drug in the medium.3 Hence, the selected promastigotes after each culture cycle were grown in gradually increasing phleomycin levels up to 150 μg/ml of drug were subjected to cell cycle analysis. Cell cycle analysis by flow cytometry showed that the arrest in growth of the LdCEN−/− promastigotes at 24 h occurred with a significant accumulation of LdCEN−/− amastigotes at 24 h occurred with a significant accumulation of nucleus with one kinetoplast (the mitochondrial genome) (27) and axenic amastigotes at the 0-, 24-, and 48-h incubation periods. The culture at the 0-h time point indeed was the exponentially growing promastigote parasites used to initiate the axenic amastigote culture for the assay. At each time point a minimum of 200 cells were counted. The data represent the means ± S.D. of three independent experiments. *, p < 0.008 Student’s t test.  

RESULTS  

Generation of LdCEN Null L. donovani Promastigotes—We homologously disrupted the two alleles of the centrin gene by homologous recombination with two different antibiotic-resistant genes as selectable markers. The hyg gene that confers resistance to hygromycin B and the neo gene that confers resistance to Geneticin were flanked at the 5′ and 3′ ends with the 5′- and 3′-UTRs of the centrin gene, respectively, and used in the recombination procedure (Fig. 1). The centrin gene status of clones was determined by Southern blot analysis using labeled gene-specific probes for centrin, hyg, and neo (Fig. 2A). The Southern blot analysis shows complete loss of the coding region of the LdCEN in double knockout mutants (Fig. 2B, lane 9). Loss of centrin expression in the LdCEN−/− parasite was also confirmed by Western blot analysis using anti-LdCen antibody (Fig. 2C, lane 3).  

Deletion of both the alleles of centrin did not affect the growth of null mutant promastigotes (Fig. 2D). The promastigote as well as axenic amastigote form of the parasite with a single centrin allele knock out (+/−) showed a growth pattern similar to that of the wild type control (+/+) (Fig. 2D, left panel). When the actively growing LdCEN−/− promastigotes (−/−) were transferred to the axenic amastigote medium, the culture showed a slight increase in cell number only in the first 24 h, a period required for the promastigotes to differentiate into axenic amastigotes. Thereafter the LdCEN−/− parasites failed to grow (Fig. 2D, right panel).  

LdCEN−/− Axenic Amastigotes Accumulate at the G2/M Stage of the Cell Cycle—To analyze the cause of the growth arrest of the axenic LdCEN−/− amastigote cells, the axenic amastigote cultures at the 24- and 48-h time points were subjected to cell cycle analysis. Cell cycle analysis by flow cytometry showed that the arrest in growth of the LdCEN−/− axenic amastigotes at 24 h occurred with a significant accumulation of cells in the G2/M phase (Fig. 3A). This accumulation became increasingly significant in cells after 48 h of culture (Fig. 3A). To further analyze the growth arrest of LdCEN−/− axenic amastigotes, 2-day-old cultures were labeled with the DNA-binding stain DAPI and observed under the fluorescence microscope. All wild type axenic amastigotes showed a single nucleus with one kinetoplast (the mitochondrial genome) (27) (Fig. 3B, upper panels). However, the LdCEN−/− axenic amastigotes showed large cells with multiple nuclei and kinetoplasts (Fig. 3B, lower panels).  

3 A. Selvapandiyan, A. Debrabant, R. Duncan, J. Muller, P. Salotra, G. Sreenivas, J. L. Salisbury, and H. L. Nakhasi, unpublished observation.
amastigotes were examined by electron microscopy (Fig. 3C). The large cells were multinucleated and typically had more than one kinetoplast. These cells were highly pleomorphic in shape as opposed to the wild type control cells that are spherical (Fig. 3C). Some of the multinucleated cells, probably the older ones, displayed condensed nuclei, a feature of cells undergoing apoptosis (data not shown). To quantitate the multinuclear status of the LdCEN\(^{-/-}\) axenic amastigotes, we counted the multinucleated cells after various days in culture, stained with DAPI by fluorescent microscopy. The analysis showed a progressive increase of multi-nucleated cells with time in axenic amastigote cultures of the centrin knockout LdCEN\(^{-/-}\) parasites incubated for 120 and 240 h. Am, amastigotes; M, multinucleated parasites. Scale bar, 10 μm.

B and C, percentage of infected macrophages (B) and number of parasites/infected macrophage (C) at various time points post-infection. The data represent the means ± S.D. of three independent experiments. *, p < 0.005; **, p < 0.0001 Student’s t test.

**Fig. 5.** Macrophage infection. A, light microscopy of the Wright-stained human macrophages infected with the LdCEN\(^{+/+}\) (+/+) and LdCEN\(^{-/-}\) (−/−) parasites incubated for 120 and 240 h. Am, amastigotes; M, multinucleated parasites. Scale bar, 10 μm. B and C, percentage of infected macrophages (B) and number of parasites/infected macrophage (C) at various time points post-infection. The data represent the means ± S.D. of three independent experiments. *, p < 0.005; **, p < 0.0001 Student’s t test.

with broadly divergent basal body associated centrin isotypes except LdCenp (12), was performed (Fig. 3E). Both wild type and LdCEN\(^{-/-}\) promastigote cells showed conspicuous localization of a centrin at the base of emergent flagella as one or two bright spots, in addition to diffuse staining along the flagellar axoneme (Fig. 3E, panels a and c). In axenic amastigotes, however, consistent localization of centrin at the flagellar base was seen only in wild type cells (Fig. 3E, panels b and d). High resolution electron microscopy of the flagellar apparatus was conducted to determine whether specific alterations in basal bodies or associated structures could be distinguished in LdCEN\(^{-/-}\) promastigote or axenic amastigote cells (Fig. 3F). Thin sections through the flagellar pocket revealed paired basal bodies in promastigote and axenic amastigote cells that were essentially indistinguishable between the wild type and LdCEN\(^{-/-}\) cells (Fig. 3F, panels a–d). However, the multiple basal bodies (three or four) readily seen in individual cells of proliferating cultures of wild type promastigote and amastigote and in LdCEN\(^{-/-}\) promastigote cells (Fig. 3F, panels e–g) could not be found in LdCEN\(^{-/-}\) axenic amastigotes, despite extensive analysis of sampled material (Fig. 3F, panel h). Together with the immunofluorescence analysis, these observations suggest that expression of centrin is required for basal body duplication in axenic amastigote cells, whereas basal body duplication in promastigote cells can proceed in the absence of LdCen, presumably through the action or compensation by other Leishmania centrin or other proteins.

LdCEN\(^{-/-}\) Axenic Amastigotes Initiate a Programmed Cell Death Pathway after Their Growth Arrest—To assess the fate of the large multinucleated LdCEN\(^{-/-}\) axenic amastigote cells, we analyzed them by PI staining and fluorescence-activated cell sorter analysis. There was a progressive increase in the number of PI-positive cells, indicating a loss of membrane integrity and cell death in LdCEN\(^{-/-}\) axenic amastigote culture over time (Fig. 4A). The LdCEN\(^{-/-}\) parasites showed at least 2-fold more PI-positive cells than the

**Fig. 6.** A, growth analysis of the axenic amastigote parasites of the LdCEN\(^{+/+}\) (+/+) and LdCEN\(^{-/-}\) (−/−) parasites incubated for 120 and 240 h. Am, amastigotes; M, multinucleated parasites. Scale bar, 10 μm. B, Western blot analysis of the cell lysates from axenic amastigotes of Leishmania +/+ (−/−), +/+/AB, and −/−/AB. The blots were developed using anti-LdCen antibody
wild type at both 24 and 48 h of culture (Fig. 4A). The death of cells after becoming multinucleated suggested that an internal mechanism had triggered a programmed cell death. To characterize the death pathway, axenic amastigotes of \textit{LdCEN}^-/^- or wild type cells were subjected to the TUNEL assay for the nicked DNA characteristic of the nuclei of apoptotic cells (24). The TUNEL assay in Fig. 4B (panels a–d) shows the fluorescent microscopic pictures of cells showing DAPI staining as red and TUNEL labeling as green. In Fig. 4B (panels e and f), the merged DAPI and the TUNEL images reveal that the \textit{LdCEN}^-/^- axenic amastigotes were TUNEL-positive. A few TUNEL-positive cells were observed in the wild type cells (Fig. 4C). In contrast, in the \textit{LdCEN}^-/^- axenic amastigotes a much greater percentage of cells showed TUNEL positivity both 24 and 48 h after shifting to amastigote medium (Fig. 4C). As further evidence for the programmed cell death pathway in centrin null mutant cells, we have observed a higher portion of PhiPhiLux-positive cells in the \textit{LdCEN}^-/^- axenic amastigotes than in the \textit{LdCEN}^+/+ parasites (data not shown). PhiPhiLux cleavage is a measure of caspase-like activity and was shown to induce in \textit{L. donovani} parasites either in response to anti-leishmanial drugs or during growth arrest (24). Thus these results suggested that the \textit{LdCEN}^-/^- axenic amastigotes undergo a programmed cell death pathway after they become multinucleated and stop growing.

\textit{LdCEN}^-/^- Parasites Do Not Survive inside Macrophages—Because centrin-deficient \textit{L. donovani} do not grow as axenic amastigotes in \textit{vitro}, we examined their survival in macrophages in \textit{vitro}. To this end, \textit{in vitro} differentiated human macrophages were inoculated with stationary phase cultures of wild type and \textit{LdCEN}^-/^- promastigotes (Fig. 5). The results at 5 h post-infection showed that the percentage of macrophages that take up the parasites was similar (>80%) with both types of parasites (Fig. 5B). These macrophage cultures were subsequently examined at 24, 48, 120, and 240 h post-infection, and the percentage of infected macrophages was calculated. After 120 h the \textit{LdCEN}^-/^- cells appeared to have differentiated into amastigotes without further growth, and multinucleated large cells were seen that resembled the multinucleated cells in culture (Fig. 5A), whereas wild type control cells did not become multinucleated in macrophages and continued to grow (Fig. 5A). The percentage of macrophages infected with \textit{LdCEN}^-/^- parasites decreased significantly to as low as 12% at 240 h (Fig. 5B), whereas, at the same time, 46% of the macrophages were infected with the control parasites (Fig. 5B). The parasite load at 240 h post-infection was significantly more (12/macrophage) for wild type parasite than for the mutant parasite (1.8/macrophage) (Fig. 5C). After 312 h post-infection, no mutant parasite was seen in the macrophages (data not shown). These results suggested that the centrin-deficient parasites do not survive in the macrophages in \textit{vitro}.

Rescue of Growth Arrest by Centrin Expression in the \textit{LdCEN}^-/^- Axenic Amastigotes—To confirm that disruption of centrin gene expression was the specific cause of growth inhibition in \textit{LdCEN}^-/^- axenic amastigotes, recombinant LdCen was episomally expressed in the knockout parasites. \textit{LdCEN}^-/^- promastigotes were transfected with the pXG-PHLEO-LdCEN plasmid and selected to grow in presence of phleomycin (150 \(\mu\)g/ml). When transferred to axenic amastigote culture conditions in the presence of phleomycin, the transfected parasite differentiated and grew as axenic amastigotes similar to wild type (WT) (Fig. 6A). Western blot analysis of the phleomycin-resistant axenic amastigotes confirmed the episo-}

**DISCUSSION**

**Defects in Basal Body Duplication and Cytokinesis in the \textit{LdCEN}^-/^- Axenic Amastigotes**—In the present study we demonstrated for the first time the requirement for centrin in basal body duplication and cytokinesis selectively only in the amastigote stage and not in the promastigote stage of \textit{Leishmania} (Fig. 7), whereas centrin was uniformly required for centriole/basal body duplication and cytokinesis in all cell types studied in both unicellular as well as multicellular organisms (1, 2, 4, 5, 8–10, 28). Several mechanisms can explain failed cell division. Basal body/centriole duplication occurs once/cell division, and failed duplication or overproduction of these organelles would lead to aberrant mitotic spindles (2, 4, 29). The exact mechanism by which centrin affects basal body duplication in eukaryotic cells including the amastigote cells of \textit{Leishmania} remains unknown. However, it is required for the duplication of basal bodies in this parasite.

As a unique feature in the Trypanosomatid members, the kinetoplast (the mitochondrial DNA disc) lies next and orthog-
programmed cell death pathway in a protozoan parasite (Fig. 7), a phenomenon observed so far only in higher eukaryotic cells when there is a loss of cell cycle control (31, 32). Thus a programmed cell death pathway in Leishmania could be triggered by an internal signal as well as the external stimuli observed previously in our laboratory (24, 33).

Stage-specific Growth Defect of LdCEN+− Parasites—It is unique and surprising to see normal basal body duplication and cell division in the promastigote stage of the LdCEN knockout parasites. We observed that the level of expression of LdCenp was similar in both promastigotes and axenic amastigotes (12). However, we also had observed the existence of more than one centrin type in the L. donovani (12). Our recent search of the *L. major* genome data bank revealed several additional *Leishmania* centrin-related genes. Therefore, it is possible that different centrin genes may have stage-specific differential expression and that one of these may complement LdCenp function only for promastigotes in the LdCEN+− parasite and not in the amastigotes (Fig. 7). Alternatively, the existence of amastigote-specific growth-regulating protein(s) that interact with centrin could fail to function in the LdCEN+− amastigotes. In yeast, CDC31 protein (yeast centrin) interacts with Kar1 protein during the initial stages of spindle pole body duplication (34). Discovery of a novel centrin-binding partner, Sfi1p, lends new insight into the functional properties of centrin (6, 7). Sfi1p protein binds multiple centrin molecules along a series of internal repeats, and the Sfi1p-centrin complex forms Ca2+-sensitive contractile fibers that function to orient or position centrosomes/basal bodies and to alter centrosome structure. Genetic analysis in yeast and *Chlamydomonas* by centrin gene knockout clearly demonstrates that centrosome/basal body duplication depends on proper Sfi1p-centrin function (see Ref. 6 for a review). Taken together these results from other organisms suggest that multiple mechanisms could be operating to control the function of centrin in *Leishmania*.

The lack of centrin expression in axenic amastigotes affected specifically their growth in vitro as well as in macrophages. Such a defect in survivability in macrophages is an indication of the lack of parasite virulence, and therefore these LdCEN-deficient parasites could be further tested as a potential vaccine against leishmaniasis. Studies of gene knockouts and their effect on *Leishmania* virulence have also been performed by several other investigators (16, 17, 35–38), although such alterations did not differentiate between the promastigote and amastigote forms of *Leishmania*. The present report describes for the first time a gene knockout for a cytoskeletal structural protein in *Leishmania* and the importance of such a gene for the growth of the parasite. Inactivation of the centrin gene in the amastigote form of related *Leishmania* species, which cause other forms of leishmaniasis, such as cutaneous and mucocutaneous diseases, will ultimately be pursued to develop attenuated strains for those species. Such attenuated strains could be vaccine candidates for these diseases as well. Vaccine candidates for other parasitic diseases, such as malaria and Chagas, also could be considered by similar inactivation of the centrin gene, which is likely to play an essential role in those organisms too.

Acknowledgments—We thank S. M. Beverley (Washington University School of Medicine, St. Louis, MO) for providing plasmids pXG-PHLEO and pX63-HYG, G. Matlashewski (McGill University, Montreal, Canada) for providing plasmid pKS-NEO, and the Department of Transfusion Medicine, National Institute of Health, for providing the elicited human monocytes.

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