Morphological Events during the Cell Cycle of *Leishmania major*

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The morphological events involved in the *Leishmania major* promastigote cell cycle have been investigated in order to provide a detailed description of the chronological processes by which the parasite replicates its set of single-copy organelles and generates a daughter cell. Immunofluorescence labeling of β-tubulin was used to follow the dynamics of the subcellular cytoskeleton and to monitor the division of the nucleus via visualization of the mitotic spindle, while RAB11 was found to be a useful marker to track flagellar pocket division and to follow mitochondrial DNA (kinetoplast) segregation. Classification and quantification of these morphological events were used to determine the durations of phases of the cell cycle. Our results demonstrate that in *L. major* promastigotes, the extrusion of the daughter flagellum precedes the onset of mitosis, which in turn ends after kinetoplast segregation, and that significant remodelling of cell shape accompanies mitosis and cytokinesis. These findings contribute to a more complete foundation for future studies of cell cycle control in *Leishmania*.

*Leishmania* spp. are protozoan parasites that are causative agents of the leishmaniases, a spectrum of vector-borne diseases endemic in tropical and subtropical countries. The cutaneous form of leishmaniasis is the most common form, and it is estimated that it afflicts about 10 million people. In Africa and Asia, this disease is caused mainly by *Leishmania tropica* and *L. major*. The two major replicative developmental stages in the life cycle of *Leishmania* are the procyclic promastigote, which occurs in the sand fly insect vector, and the amastigote, which resides in the phagolysosome of mammalian macrophages. *Leishmania* procyclic promastigotes are highly polarized cells that possess a number of single-copy organelles with defined subcellular locations. These include the nucleus, the Golgi apparatus, the basal body, the mitochondrion (which incorporates the kinetoplast), and the flagellum, which protrudes from the cell body via the flagellar pocket. The generation of viable progeny relies upon precise control of the duplication and segregation of these organelles (11, 17, 29, 32).

The cell cycle of procyclic form *Trypanosoma brucei* has been characterized extensively and forms a basis for comparison with other trypanosomatids, including *Leishmania*. Notably, in *T. brucei*, replication of the nuclear and kinetoplast DNAs (S phase) starts almost synchronously, whereas the division and segregation periods for the nucleus (M and C phases, respectively) and kinetoplast (D and A phases, respectively) are separated in time. In procyclic form *T. brucei* as well as in *L. tarentolae* (28), kinetoplast division is completed before the onset of nuclear mitosis, while in *L. mexicana* and *L. donovani* these events appear to occur in the reverse order (11, 17, 32). The chromosomes of *T. brucei* do not visibly condense, the nuclear envelope remains intact during mitosis, and no structural equivalents of the mammalian spindle pole body poles (centrosomes) have been identified (19). However, the centrioles of the flagellar basal bodies are involved directly in the partitioning of the mitochondrial genome (23), and chromosome segregation in the dividing nucleus involves not only kinetochores but also interpolar microtubules (7, 8). The extensive remodelling of the microtubular cytoskeleton that occurs during mammalian cell cytokinesis has not yet been described for any trypanosomatid, although Wheeler et al. (32) detailed changes in the cell shape of promastigotes of *L. mexicana* from entry into mitosis to early cytokinesis. Also, the mechanisms involved in the furrowing of the plasma membrane of trypanosomatids leading to cell abscission are still obscure (10).

Microtubules, however, are integral to many trypanosomatid structures: the cytoplasmic microtubules of the subpellicular corset reinforce the plasma membrane and define the shape of the cell; the flagellar microtubules form the axoneme; the intranuclear microtubules compose the mitotic spindle; and microtubules are densely packed, in proximity to the kinetoplast, in structures such as the basal bodies (20). Moreover, it is well established that in these parasites, progression throughout the cell cycle is dependent on microtubule-mediated events, and interfering with microtubule polymerization/denpolymerization has been shown to affect basal body duplication, kinetoplast segregation, flagellar axoneme growth, mitosis, cell growth, and cytokinesis of trypanosomes (16, 22–24, 35). Microtubule-mediated events also play major roles in progression of the *Leishmania* cell cycle (4, 12, 13), and treatment of *Leishmania* promastigotes with antimicrotubule agents re-
sulted in inhibition of nuclear mitosis and cytokinesis and inaccurate positioning of the kinetoplast within the cell (11).

Leishmania and Trypanosoma are closely related trypanosomatids; nevertheless, there are significant morphological differences between them that have implications for the way in which they divide. Leishmania promastigotes possess a single flagellum that emerges from the anterior portion of the cell body, while the flagellum of T. brucei is attached for most of its length to the convex edge of the cell body via the flagellar attachment zone (FAZ), a structure anchored to the subpellicular microtubule array through a specific microtubule quartet. Even though Leishmania organisms do not possess an FAZ, this microtubule quartet is conserved and is found surrounding the length of the cell body, and it has been postulated to be involved in the maintenance of the multivesicular tubule (MVT) lysosome (18, 31). Many single-copy organelles are concentrated in the part of the cell where the flagellar pocket is located; for Leishmania promastigotes, this is at the anterior end, whereas for T. brucei procyclic form parasites, it is in the posterior region. The latter extensity of T. brucei is the site of extensive microtubule polymerization (24, 30) and of cell elongation during the growth phase of the cell cycle (27), while the anterior end is the point from which cytokinesis starts and progresses.

The lack of a comprehensive description of the Leishmania cell cycle prompted us to carry out a detailed morphological analysis of the processes occurring during the L. major procyclic promastigote cell cycle. In addition to standard microscopic observations, we used immunofluorescence labeling of the subpellicular microtubules, mitotic spindle, and flagellar pocket to allow us to determine accurately the different phases of the cell cycle. The timing of the L. major cell cycle is similar to that for T. brucei (35) and Trypanosoma cruzi (5), despite the morphological differences between these parasites. In addition, our data allow a direct comparison with a recently published analysis of the L. mexicana cell cycle (32).

**MATERIALS AND METHODS**

**Cultivation of Leishmania major promastigotes.** Leishmania major (MHOM/IL/80/Friedlin) promastigotes, recently differentiated from amastigotes taken from mice, were grown in HOMEM (a modified Eagle’s medium; Invitrogen) with 10% (vol/vol) heat-inactivated fetal calf serum (HIFCS) at 25°C. Experiments were performed on cells in the logarithmic phase of growth (0.3 × 10^7 to 0.7 × 10^7 cells/ml).

**Expression of GFP-RAB11 in L. major.** The L. major RAB11 gene (LmjF10.0910) was amplified by PCR from L. major genomic DNA and cloned into the pNUS-GFP vector for expression with an N-terminal green fluorescent protein (GFP) tag. Transfection was performed with an Amaxa Nucleofector kit for human T cells, following the manufacturer’s instructions. Briefly, 5 × 10^7 promastigotes in the mid-log phase of growth were resuspended in 100 μl of Amaxa Nucleofector buffer and mixed with 10 μg of DNA. Cells were electroporated using the Amaxa U-033 program before being transferred to 10 ml of fresh HOMEM with 20% (vol/vol) HIFCS. Transfectants were obtained under G418 (15 μg/ml) selection. Live cells were incubated with DAPI (4',6-diamidino-2-phenylindole) at a concentration of 1 μg/ml before being washed and resuspended in ice-cold phosphate-buffered saline (PBS) for immediate analysis.

**Immunofluorescence.** L. major promastigotes (10^7) were washed twice in PBS before fixation in 200 μl of 1% formaldehyde in PBS for 30 min at room temperature (RT). After a PBS wash, the cells were then permeabilized by resuspension in 200 μl of 0.1% Triton X-100 in PBS for 10 min. Following an additional PBS wash, the cells were resuspended in 200 μl of 0.1 M glycine in PBS and incubated for a further 10 min at RT before being washed in PBS. Glass slides were washed with 70% (vol/vol) ethanol and coated with a 0.01% solution of poly-L-lysine (0.1% stock, Sigma), and the fixed, permeabilized cells were then left to sediment and adhere to the surfaces of these polylysine-coated slides for 15 min at RT. Monolonal mouse anti-β-tubulin IgG2b (KMX1) (3) and/or polyclonal rabbit anti-T. brucei RAB11 (anti-TbRAB11) (14), diluted 1:1,000 in 0.1% (vol/vol) Triton X-100, 0.1% (wt/vol) bovine serum albumin (BSA), and PBS (TB buffer), was added to the slide and incubated with the cells for 2 h at RT. After a 10-ml PBS wash, cells were incubated in the dark for 1 h at RT with Alexa Fluor 488 (green)-conjugated or Alexa Fluor 594 (red)-conjugated secondary antibodies (Molecular Probes) diluted 1:1,000 in TB buffer. The cellular DNA was then stained with 0.5 μg/ml DAPI (Sigma) for 1 min before proceeding to a final 10-ml PBS wash at RT. A mounting solution (2.5% 1,4-diazobicyclo[2.2.2]octane [DABCO] in 50% (vol/vol) glycerol) was ultimately applied to the slide. Cells were viewed with a Zeiss UV microscope, and images were captured with an Orca-ER camera (Hamamatsu) and Openlab software, version beta. Images were prepared for presentation using Adobe Photoshop CS.

**ConA labeling.** A total of 0.5 × 10^7 early-logarithmic-phase L. major promastigotes were sedimented at 1,000 × g for 5 min at 4°C and washed once in serum-free HOMEM. Cells were then incubated in serum-free HOMEM with 50 μg/ml of fluorescein isothiocyanate (FITC)-conjugated concanavalin A (ConA; Invitrogen) for 1 h at 4°C. After two washes in PBS at 4°C, cells were finally fixed and prepared for immunofluorescence analysis as described above.

**FM4-64 labeling.** FM4-64 labeling was performed as described previously (2). Briefly, 0.5 × 10^7 live L. major promastigotes expressing GFP-RAB11 were pelleted (1,000 × g, 5 min, 4°C), resuspended in serum-free HOMEM containing 40 μM FM4-64 (Molecular Probes), and incubated for 15 min at 4°C. DAPI was then added to a final concentration of 1 μg/ml and incubated at RT for 2 min. After being washed twice in ice-cold PBS (by centrifugation at 1,000 × g for 2 min at 4°C), parasites were applied to slides for immediate viewing by microscopy. Images were captured using a Delta Vision RT deconvolution microscope system (Applied Precision). DAPI, GFP, and FM4-64 were imaged with 480-nm, 540-nm, and 580-nm filters, respectively.

**Cell cycle analysis.** For the classification and quantification of morphological configurations by using DAPI and anti-β-tubulin staining, a total of 1,500 promastigotes were counted in 3 independent experiments. Williams analysis (33) was used to determine the duration of the M and C phases for the nucleus and the D and A phases for the kinetoplast, using the frequencies of cells within the population exhibiting relevant DAPI configurations, as described previously (35). This uses the formula \( x = \ln(1 - y/2)/\mu \), where \( x \) is the cumulative time within the cycle taken to reach the end of the morphological stage in question, \( y \) is the cumulative % of cells up to and including the stage in question (expressed as a fraction), and \( \mu \) is the specific growth rate, determined as follows (where PDT is the population doubling time): \( \mu = \ln(2)/PDT \). Exponentially growing L. major promastigotes were seeded at 3 × 10^5 cells/ml, and cell density was estimated using a hemocytometer at hourly intervals. A growth curve was constructed, and the PDT was calculated.

**Western blot analyses.** Promastigotes were lysed in 2% SDS with a cocktail of peptidase inhibitors (0.1 mg/ml leupeptin, 1 mM phenanthroline, 0.5 mg/ml Pefabloc SC, 5 mg/ml pepstatin A, and 0.1 mg/ml phenylmethylsulfonyl fluoride, all purchased from Sigma-Aldrich). For fractionation experiments, cells were homogenized in 0.5% Nonidet P-40 in PEBME buffer [100 mM piperazine-2-ethanesulfonic acid (PIPES), pH 6.9, 2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA] on ice for 5 min (modified from references 23 and 34). The preparations were then centrifuged at 250 × g for 30 min at 4°C. The soluble fraction (S) was collected for analysis. The insoluble pellet was washed twice in PEBME buffer before being resuspended in 0.5% NP-40–PEBME buffer (insoluble fraction [I]). Whole-cell and fractionated cell lysates were resolved using 12% (wt/vol) SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and immunoblotted with anti-T. brucei RAB11 antibody (14) diluted 1:1,000 and 1:10,000, respectively, for the two types of lysates) in PBS with 5% (wt/vol) skim milk and 0.05% Tween 20. For the fractionation experiments, the membranes were also incubated with anti-T. brucei β-tubulin (KMX1; 1:10,000) (3). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody and the SuperSignal West Pico chemiluminescence system (Pierce) were then used to visualize the marked antigens.

**RESULTS**

One of the most effective and quickest ways to identify dividing cells among a population of asynchronous L. major promastigotes is to observe morphological changes by phase-contrast microscopy. Under these conditions, the outgrowth of a second flagellum (F) constitutes one of the earliest identifiable events in cell division. Taken together with DAPI staining
of the nucleus (N) and kinetoplast (K), this allowed us to define the cell cycle position of individual cells within a population by organelle number; for example, a cell in G₁ phase has a 1N1K1F configuration, and a cell undergoing cytokinesis has a 2N2K2F configuration. In order to define more precisely the progression of kinetoplast segregation and nuclear mitosis in *L. major*, we searched for other markers of these processes. An antibody raised against *T. brucei* RAB11 which also cross-reacts with the *L. major* homologue (Fig. 1; see Fig. 3) was unexpectedly found to have utility in the visualization of flagellar pocket division and thus kinetoplast segregation in *L. major* (designated 1K¹) (see Fig. 3 and 4).

**Immunofluorescence labeling of β-tubulin during the *L. major* cell cycle.** We used β-tubulin staining with the KMX1 monoclonal antibody as a marker to help define cell cycle progression. In interphase promastigotes (1N1K1F), the anti-β-tubulin antibody labeled mainly the subpellicular microtubules (Fig. 2A). For cells possessing a wider cell body, the posterior-end microtubules were predominantly labeled (1N1K1F) (Fig. 2B). Upon the initiation of daughter flagellum elongation and at the onset of mitosis, the β-tubulin signal was present mainly at the center of the nucleus (Fig. 2C, white arrowhead). We classified these cells as 1NM1K2F cells. The size of the intranuclear structure increased as mitosis progressed, until the parent and daughter DNAs started to migrate apart and the structure elongated (Fig. 2D). A characteristic mitotic spindle was then observed (Fig. 2E and F). This structure formed mainly along the longitudinal axis of the dividing nucleus (Fig. 2D), thus being perpendicular to the kinetoplast, and its elongation during progression into mitosis was accompanied by a change in cell shape such that, by mid-mitosis, the cell had gone from an elongated to a rounded cell.

![Western blot of *L. major* promastigote cell extract with anti-β-tubulin KMX1 and anti-RAB11 antibodies. Promastigote cells were lysed in 0.5% NP-40, fractionated by centrifugation at 250 g for 30 min into a soluble fraction (S) and an insoluble fraction (I), blotted, and probed with *T. brucei* anti-RAB11 and KMX1 antibodies. Two forms of *L. major* RAB11 were detected (black and red arrowheads).](image1)

**FIG. 1.** Western blot of *L. major* promastigote cell extract with anti-β-tubulin KMX1 and anti-RAB11 antibodies. Promastigote cells were lysed in 0.5% NP-40, fractionated by centrifugation at 250 g for 30 min into a soluble fraction (S) and an insoluble fraction (I), blotted, and probed with *T. brucei* anti-RAB11 and KMX1 antibodies. Two forms of *L. major* RAB11 were detected (black and red arrowheads).

![Immunofluorescence analysis of β-tubulin during the *L. major* cell cycle. Fixed cells were labeled with mouse KMX1 anti-β-tubulin monoclonal antibody and Alexa 488-conjugated anti-mouse antibody (green). The nuclear and kinetoplast DNAs were stained with DAPI (blue). (A to I) Representative pictures of the 1,500 cells examined are shown. (Left) DIC images. (Right) Merged images of β-tubulin (green) and DAPI (blue) staining. The white arrowheads indicate the microtubules of the mitotic spindle. The white arrows indicate an area of constriction where β-tubulin accumulates along the division plane. The black arrowheads indicate the presence of a growing daughter flagellum. Bar = 10 μm.](image2)

**FIG. 2.** Immunofluorescence analysis of β-tubulin during the *L. major* cell cycle. Fixed cells were labeled with mouse KMX1 anti-β-tubulin monoclonal antibody and Alexa 488-conjugated anti-mouse antibody (green). The nuclear and kinetoplast DNAs were stained with DAPI (blue). (A to I) Representative pictures of the 1,500 cells examined are shown. (Left) DIC images. (Right) Merged images of β-tubulin (green) and DAPI (blue) staining. The white arrowheads indicate the microtubules of the mitotic spindle. The white arrows indicate an area of constriction where β-tubulin accumulates along the division plane. The black arrowheads indicate the presence of a growing daughter flagellum. Bar = 10 μm.
body (Fig. 2, compare panels C and F). Upon completion of mitosis, the KMX1 anti-β-tubulin antibody labeled predominantly the subpellicular microtubules. By this stage, the divided nuclei were positioned laterally within the cell, thus ensuring their distribution on both sides of the division plane and their accurate allocation to the parent and daughter cells (Fig. 2G, 1N*1K*2F cells). With the onset of cytokinesis, in some 1N*2K*2F cells but mostly in 2N2K2F cells, the KMX1 anti-β-tubulin antibody labeled mainly the site of cleavage furrow ingression (Fig. 2G, H, and I, white arrows). The main β-tubulin signal then appeared to follow the progression into cytokinesis, becoming more intense in the tightening region up to cell-cell scission (Fig. 2I).

Immunoﬂuorescence labeling of RAB11 during the L. major cell cycle. The subcellular localization of RAB11 was analyzed in L. major by using an antibody raised against T. brucei RAB11 (TbRAB11); this was found to be a useful marker of flagellar pocket separation and thus of kinetoplast segregation. In interphase cells, the anti-RAB11 antibody labeled a structure at the base of the flagellum, along with a “two-pronged fork” structure positioned between the first structure and the kinetoplast (Fig. 3A). This observation was most surprising, as no other endosomal compartment characterized so far in trypanosomatids has a similar shape. Colocalization with ConA, which labels the Leishmania surface, including the flagellar pocket, before being endocytosed (1), showed that some of the RAB11-positive compartment was in close association with the flagellar pocket (Fig. 3A, arrowhead fp), while the two-pronged fork structure was in close proximity to ConA-labeled endosomes (Fig. 3A, arrowhead e). This RAB11-positive compartment was also closely associated with the kinetoplast, and its morphology evolved in parallel with the kinetoplast division cycle (see Fig. 4).

A gene encoding a putative RAB11 protein (LmjF10.0910) that shares 78% amino acid sequence identity with TbRAB11 was identified in the L. major genome. Western blot analyses using the anti-TbRAB11 antibody allowed us to detect the putative RAB11 orthologue of L. major, revealing a protein of the predicted size (23.3 kDa) along with an additional, slower-migrating protein that may represent a geranylgeranylated form, as described for T. cruzi (15) (Fig. 1, red and black arrowheads, respectively). An L. major cell line expressing GFP-LmjRAB11 was generated and used to demonstrate that the anti-TbRAB11 antibody recognized both the L. major GFP-RAB11 fusion protein and the endogenous RAB11 protein (Fig. 3B, green and red arrowheads, respectively). Live-cell imaging of the GFP fusion protein revealed a close association of GFP-RAB11 with the kinetoplast and flagellar pocket, similar to that detected with the anti-RAB11 antibody (Fig. 3C to F). This was conﬁrmed by partial colocalization of GFP-RAB11 with FM4-64, a ﬂuorescent lipophilic tracer known to accumulate in the flagellar pocket when cells are incubated at 4°C (2) (Fig. 3E and F). We were thus conﬁdent that the anti-TbRAB11 antibody recognizes L. major RAB11, so immunofluorescence experiments were performed with L. major promastigotes (Fig. 4).

During the early events of the cell cycle, the RAB11-positive compartment could be visualized as a two-pronged fork-like form (Fig. 4A) or as a single strand (Fig. 4B), likely depending on the plane of view. As the parasites progressed through the cell cycle, the shape of the RAB11-positive compartment developed. In cells in which a second flagellum was beginning to emerge, an additional RAB11-positive structure could be identiﬁed next to the existing one, indicating the generation of a daughter flagellar pocket and the start of kinetoplast division (Fig. 3F and 4C and D). The appearance of this second RAB11-positive flagellar pocket proved to be a useful tool for monitoring the start of D phase. During this process, a slight rotation of the kinetoplast on its axis was frequently observed, such that it appeared somewhat tilted to one side (Fig. 4D). The RAB11-positive structure progressively extended from the newly synthesized flagellar pocket until ultimately both the old and new compartments appeared to be associated with the same elongated kinetoplast (Fig. 4E). It appeared that the extension of the daughter flagellum was associated with the development of the newly synthesized RAB11 fork structure as well as with the separation of the two flagellar pockets and the segregation of the kinetoplast (Fig. 4F).

Chronology of morphological events leading to Leishmania division. To allow us to generate a detailed description of the cell cycle of L. major, all morphological conﬁgurations identiﬁed in an asynchronous population of cells in the logarithmic phase of growth were classiﬁed and quantiﬁed. Nucleus and kinetoplast conﬁgurations were counted after DAPI staining, and the number of flagella was assessed from differential interference contrast (DIC) images. In addition, the dynamics of the β-tubulin- and RAB11-positive compartments were followed to facilitate the identiﬁcation of mitotic cells and those with a dividing kinetoplast.

Two conﬁgurations of 1N1K1F cells could be identiﬁed based on staining with β-tubulin. For cells with a long, thin body, the KMX1 antibody labeled the whole array of subpellicular microtubules (Fig. 5A, conﬁguration a), while for cells with a wider body shape, the antibody labeled mainly the posterior end of the cell (Fig. 5B, conﬁguration a’). The presence of a daughter flagellum is the earliest indication that a new round of cell division has initiated, and this was recently shown in L. mexicana promastigotes (32) to be indicative that DNA replication has occurred. To reﬁne the nomenclature, we used an asterisk to deﬁne a nucleus (1N*) or kinetoplast (1K*) in the process of DNA replication (S phase) or in G2 phase prior to segregation. In 1N*1K*2F cells, little subpellicular staining of the β-tubulin could be observed (Fig. 5C, conﬁguration b). This might be due to preferential afﬁnity of the KMX1 antibody for newly assembled Leishmania β-tubulin that has not been modiﬁed posttranslationally. This β-tubulin may still posses its C-terminal tyrosine, in contrast to older, detyrosinated β-tubulin (25). According to this hypothesis, the sites of microtubule assembly and the recently formed tubulin would be labeled preferentially. Consistent with this hypothesis was the observation, in some 1N1K1F cells, of preferential KMX1 antibody staining of the posterior end, known to be the site of the microtubule structural assembly. Also, at the onset of mitosis, the KMX1 antibody preferentially detected β-tubulin in the forming mitotic spindle in cells which also harbored a duplicated RAB11-positive compartment, and these were thus identiﬁed as 1N*1K*2F cells (Fig. 5D to G, conﬁgurations c, d, and e). In these cells, the elongation of the intranuclear mitotic spindle initially followed a longitudinal axis (Fig. 5D and E, conﬁguration c) and was later accompanied by a rotational
movement (Fig. 5F, configuration d) that resulted in the lateral positioning of the dividing nucleus (Fig. 5G, configuration e). The mitotic spindle was found to be present after segregation of the kinetoplast, as 1NM2K2F cells (Fig. 5H, configuration f) but no 2N1K2F cells were observed, thus indicating that in *L. major* promastigotes, kinetoplast division finishes before completion of mitosis. These data highlight the importance of following the distribution of β-tubulin in the cell in addition to counting the number of nuclei by DAPI staining, as this allowed the accurate discrimination of cells containing a mitotic nucleus that might appear to contain two separate nuclei by DAPI staining alone (Fig. 5F, G, and H, configurations d, e, and f) from cells containing separated nuclei (Fig. 5I, configuration g). In cells that had completed the separation of parent and daughter DNA contents (categorized as 2N2K2F cells), the β-tubulin signal was again present mainly at the subpel-
icular microtubules, initially staining their positive ends as the distance between the two flagellar pockets increased (Fig. 5I, configuration g). The KMX1 antibody also marked the site of cleavage furrow ingression (Fig. 2G and H) up to cell-cell abscission (Fig. 2I and 5J and K, configuration h). In most cases, cleavage furrow ingression started at the anterior end of the cell (Fig. 5J, configuration h). Finally, rarely observed configurations, for example, cells in doublets as shown in Fig. 5G, were classified as “others” and were included in the study to further complete the classification.

As shown in Fig. 6, the combination of DAPI, β-tubulin, and RAB-11 staining allowed the precise positioning of a given cell within its cell cycle. It was thus established that in a population of asynchronous L. major promastigotes in the logarithmic phase of growth, 73.3% were in G$_1$ and/or pre-S phase/early S phase (1N1K1F), 8.5% were in the late S-G$_2$ phase of both their nucleus and kinetoplast cycles (1N$^*$1K$^*$2F) or had started their kinetoplast division already (1N$^*$1K$^*$2F), 8.4% were undergoing nuclear mitosis and kinetoplast division (1N$^*1K^*2F$), 2.2% still harbored a mitotic spindle although the kinetoplast apportioning phase had been reached (1N$^*2K^*2F$), and 4.9% had divided both their nuclear and kinetoplast DNAs and were undergoing cytokinesis (2N2K2F). Cells with minority configurations are detailed in Fig. 6B. Among the 2.7% of cells classified as “others” were cells in doublets or clustered in groups; these were not considered in the cell cycle phase duration calculations.

**Durations of the various phases of the L. major cell cycle.**
From the set of data described above, we were able to determine the durations of nuclear mitosis (M) and cytokinesis (C) as well as kinetoplast division (D) and apportioning (A) (when the nondivided cell contains two kinetochores) (Fig. 7; Table 1). This was achieved using Williams analysis (33), in which the proportion of cells containing a particular morphological configuration is taken into account along with the specific growth rate of the culture to determine the length of time taken to reach the end of the stage in question (see Materials and Methods for a full description). Based on this analysis, L. major nuclear mitosis and cytokinesis take 1.4 h and 1.1 h, respectively, while kinetoplast division and apportioning take 2.1 h and 1.4 h, respectively, for cell cultures with an estimated doubling time of 10.2 h.

These data are summarized in Table 1, with data determined for the cell cycles of T. brucei, T. cruzi, and L. mexicana also included for comparison (5, 32, 35).

**DISCUSSION**

The cell cycle studies presented here were performed on in vitro cultures of L. major promastigotes, recently differentiated from amastigotes isolated from an infected mouse, in the log-
arithmic phase of growth. The percentage of dividing cells (defined as cells with duplicated DNA content) within the promastigote population was 15.5%. This is similar to findings with *L. donovani*, for which it was reported that under normal growth conditions, 10 to 20% of the cells were dividing (11), and *L. mexicana*, for which about 10% of the cells had a divided nucleus and/or kinetoplast (32). Although the data for the three species were obtained under different experimental conditions, they do suggest that promastigotes of the three *Leishmania* species behave similarly in culture. Thus, the new insights revealed in the detailed analysis of cell cycle progression in *L. major* promastigotes presented here are very likely to be relevant to other *Leishmania* species.

The ultrastructure of the dividing nucleus in *T. brucei* has been studied in detail by electron microscopy and immunofluorescence analysis of the mitotic spindle labeled with the KMX1 anti-β-tubulin antibody (19). We also took advantage of the KMX1 anti-β-tubulin antibody to label the mitotic spindle in *L. major* promastigotes in order to distinguish between dividing (1N\textsuperscript{M}) and divided (2N) nuclei. Our study showed that while at the onset of mitosis the majority of the *Leishmania* cells had a longitudinal mitotic spindle, once mitosis was completed, most of the parent and daughter nuclei were arranged laterally within the dividing cell. Therefore, it seems likely that the latter orientation results from rotation of the two nuclei. This movement apparently takes place mainly during mitosis, as all of the rotating nuclei possessed a mitotic spindle (Fig. 5 and 6, configura-
This asymmetry in nuclear division was recently also described for *L. mexicana* promastigotes (32). It is noteworthy that there are radical body shape changes around the time of nuclear mitosis. It seems likely that these morphological modifications might reflect the spatial reorganization necessary for nuclear repositioning. Similar observations were recently reported for *L. mexicana* promastigotes, where cells adopted a “scalene spheroid” morphology (32) from mitosis entry to early cytokinesis. In *T. brucei* procyclic form parasites, mitosis not only achieves chromosome segregation but also drives the correct positioning of the daughter nucleus in the gap between the parent and daughter kinetoplasts, concomitant with cell extension (24). Indeed, the intranuclear mitotic spindle was postulated to be involved in the correct positioning of the daughter nucleus via its possible anchoring to the nuclear membrane (which remains intact during mitosis) and the anterior basal body (24). In addition, it was observed that after duplication and segregation of the organelles, the replicated set of organelles rotated around the parent one, facilitating the linear arrangement of the mitochondrion-kinetoplast-basal body-flagellum complex on both sides of the unidirectional division plane (24). It is possible that a similar intricate structure connecting the nucleus, kinetoplast, basal body, and flagellum exists in *Leishmania* and participates, before initiation of cytokinesis, in the correct positioning of these organelles.

The RAB11 staining approach used in our study, allowing visualization particularly of the flagellar pocket, enabled us to visualize the timing of daughter flagellar pocket formation and to observe that, as in *T. brucei* (23), the new flagellum emerged from the cell when the parent and daughter flagellar pockets were still a single, connected structure (Fig. 5C, configuration b). This emergence of the cell-external portion of the new flagellum was found to occur near the end of S phase in *L. mexicana* promastigotes (32), supporting our assumption that cells growing a new flagellum are in S or G2 phase (1N*1K*2F). The progressive extension of the new flagellum then appeared to drive further the segregation of the parent and daughter flagellar pockets and, ultimately, the partitioning of the duplicated kinetoplast (Fig. 5H, configuration f).
Intimately linked to the formation of the daughter flagellar pocket is the segregation of the duplicated kinetoplast, which in most of the *L. major* promastigotes was complete before the end of mitosis. This finding differs from previous observations of *Leishmania* promastigotes, which indicated that the division of the kinetoplast occurred mainly after the partitioning of the daughter and parent nuclei (11, 17, 29, 32). These previous studies involved *L. mexicana* and *L. donovani*, and it is possible that the order in which kinetoplast division and mitosis happen varies between different species. However, another possible explanation for the differences between the findings is that the previous studies were based solely on observations of DAPI-stained parasites; with such an approach, it is less easy to distinguish between an elongated nucleus that has not completed the division process (1N*M*) and divided nuclei (2N).

Identification of a population of cells as 2N1K2F instead of 1N*M1K2F cells would lead to the conclusion that kinetoplast division occurs after mitosis. We believe that our experimental approaches including visualization of the mitotic spindle allow a more reliable judgment of whether mitosis was or was not completed. Our results show that in *L. major*, as in *T. brucei* (19), the disassembly of the mitotic spindle seems to be concomitant with the end of nuclear division, while in most mammalian cells remnants of the mitotic spindle are still present after completion of karyokinesis (26). Furthermore, the observation that cytokinesis was initiated mainly at the anterior part of the cell, between the two flagella, and followed a longitudinal axis toward the posterior end of the cell fits well with a model of kinetoplast segregation occurring before the end of mitosis.

Overall, the succession of events leading to the division of the kinetoplast and nucleus in *L. major* resembles that observed in *T. brucei*. A major difference, however, was observed during cytokinesis. While in both parasites cytokinesis progresses longitudinally, in *T. brucei* it involves the ingestion of a cleavage furrow that progresses in a helical fashion to adapt to the presence of the attached flagella, while in *Leishmania* promastigotes cytokinesis proceeds via an as yet uncharacterized mechanism preceded by cell rounding (Fig. 5I) (32).

Another interesting finding was the localization of RAB11 within *L. major* promastigotes. In the *T. brucei* procyclic form, RAB11 localizes to one or two defined spots, present in close proximity to the kinetoplast, that migrate with the basal body during cytokinesis (14). Since the segregation of the flagellar pockets is dependent upon basal body separation and the microtubule cytoskeleton, these differences might reflect the fact that in *T. brucei* the flagellar pocket occurs in an area of actively polymerizing microtubules, while in *Leishmania* it is located close to the minus end of the microtubules.

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**TABLE 1. Duration of cell cycle with respect to nuclear events**

| Stage | Proportion (time) of cell cycle |
|-------|-------------------------------|
|       | *L. major* (10.2-h cell cycle) | *L. mexicana* (7.1-h cell cycle) | *T. brucei* (8.5-h cell cycle) | *T. cruzi* (24-h cell cycle) |
| G₁    | 0.75 (7.7 h) (G₁ + S + G₂)    | 0.38 (2.7 h)               | 0.40 (3.4 h)               | 0.45 (10.8 h)               |
| S     | 0.4 (2.9 h) (S + G₂)          | 0.18 (1.5 h)               | 0.22 (1.8 h)               | 0.36 (8.6 h)               |
| G₂    |                               | 0.22 (1.8 h)               | 0.22 (1.8 h)               | 0.36 (8.6 h)               |
| M     | 0.14 (1.4 h)                  | 0.17 (1.2 h)               | 0.08 (0.7 h)               | 0.02 (0.4 h)               |
| C     | 0.11 (1.1 h)                  | 0.05 (0.4 h)               | 0.12 (1.1 h)               | 0.07 (1.8 h)               |

* Cell cycle phases determined in this study are compared with data for the *L. mexicana* promastigote, *T. brucei* procyclic, and *T. cruzi* epimastigote forms (5, 32, 35).
endosomal/lysosomal compartment, and disc-shaped exocytic carrier vesicles which fuse with the flagellar pocket membrane (6). T. cruzi epimastigotes possess, in addition to the flagellar pocket, a structure involved in nutrient endocytosis, named the cytostome, which is connected to a vesicular-tubular network of early endosomes (the cytopharynx) that ultimately deliver their contents to reservosomes for storage. RAB11 is present in a prelysosomal compartment composed of multiple reservosomes (15). Although Leishmania parasites appear to lack a cytostome and reservosomes, and though recycling endosomes have not yet been characterized for these parasites, their RAB11-positive compartment consists mainly of a two-pronged fork structure. This endosomal compartment extends from the flagellar pocket toward the kinetoplast, and its replication is intimately linked to that of the flagellar pocket. The explanation for the RAB11-positive compartment having such a two-pronged fork tubular structure remains unknown, but it might result from its association with the microtubule quartet involved in the structural architecture of the MTV lysosome, or it might arise from the partial duplication of the RAB11-positive compartment. The observed two-pronged fork structure might also result from the spatial hindrance caused by the tripartite attachment complex (TAC). This has been characterized for T. brucei but not previously reported for L. major. It is a complex composed of a series of filaments that connects the kinetoplast DNA network to the mitochondrial membrane and to the basal body from which the flagellum originates (20). The TAC constitutes a mechanical link by which kinetoplast segregation is associated with the division of the basal body and the flagellum. Thus, further analyses are needed to address this point and, most importantly, to identify clearly the nature and function of the RAB11-positive compartment in Leishmania promastigotes.

In conclusion, this study has provided a detailed description of the morphological events associated with progression of L. major through its cell cycle. Thus, it contributes to a more complete foundation for future studies of cell cycle control in Leishmania.

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