Centrins, Cell Cycle Regulation Proteins in Human Malaria Parasite Plasmodium falciparum*†

Babita Mahajan‡, Angamuthu Selvapandiyan‡, Noel J. Gerald‡, Victoria Majam‡, Hong Zheng‡, Thilan Wickramarachchi§, Jawahar Tiwari§, Hisashi Fujioka*, J. Kathleen Moch†, Nirbhay Kumar‡‡, L. Aravind††, Hira L. Nakhasi‡, and Sanjai Kumar‡‡

From the ‡Divisions of Emerging and Transfusion Transmitted Diseases and †Division of Biostatistics, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852, the ‡Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106, the ‡Department of Immunology, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, the **Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Baltimore, Maryland 21205 and ††National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland 20892

Molecules and cellular mechanisms that regulate the process of cell division in malaria parasites remain poorly understood. In this study we isolate and characterize the four Plasmodium falciparum centris (PfCENs) and, by growth complementation studies, provide evidence for their involvement in cell division. Centris are cytoskeleton proteins with key roles in cell division, including centrosome duplication, and possess four Ca2⁺-binding EF hand domains. By means of phylogenetic analysis, we were able to decipher the evolutionary history of centris in eukaryotes with particular emphasis on the situation in apicomplexans and other alveolates. Plasmodium possesses orthologs of four distinct centrin paralogs traceable to the ancestral alveolate, including two that are unique to alveolates. By real time PCR and/or immunofluorescence, we determined the expression of PfCEN mRNA or protein in sporozoites, asexual blood forms, gametocytes, and in the ooycts developing inside mosquito mid-gut. Immunoelectron microscopy studies showed that centrin is expressed in close proximity with the nucleus of sporozoites and asexual schizonts. Furthermore, confocal and widefield microscopy using the double staining with centrin and actin antibodies strongly suggested that centrin is associated with the parasite centrosome. Following the epistolary expression of the four PfCENs in a centrin knock-out Leishmania donovani parasite line that exhibited a severe growth defect, one of the PfCENs was able to partially restore Leishmania growth rate and overcome the defect in cytokinesis in such mutant cell line. To our knowledge, this study is the first characterization of a Plasmodium molecule that is involved in the process of cell division. These results provide the opportunity to further explore the role of centris in cell division in malaria parasites and suggest novel targets to construct genetically modified, live attenuated malaria vaccines.

Malaria, caused by infections of the intracellular parasitic apicomplexans belonging to the genus Plasmodium, exerts a significant mortality, morbidity, and economic burden in the developing parts of the world. The Plasmodium life cycle involves a vertebrate host and a mosquito host during which period the malaria parasites, for several times, undergo a significant amount of cell division and population amplification. In a vertebrate host, depending on the Plasmodium species, within a period of 2–14 days in liver cells a single sporozoite may replicate into 10,000–40,000 asexual liver-form merozoites. The merozoites invade erythrocytes, and during each intraerythrocytic replication cycle, a uninucleated merozoite can produce between 18 and 36 blood form parasites in a 24–72-h period. A small sub-set of these asexual forms differentiate into nonreplicating sexual gametocytes, a stage absolutely critical for malaria transmission through mosquitoes. In mosquito, a zygote formed by fertilization between a male and a female gamete may give rise to ~3000 infectious sporozoites. This ability to undergo abundant amplification cycles within a brief time span is critical for the survival and further transmission of malaria parasites. Although our knowledge regarding the cellular and molecular events involved in cell division in malaria parasites remains obscure, a few studies suggest that distinct cell division mechanisms regulate the multiplication of malaria parasites during different life cycle stages (1).

In eukaryotes, during the nondeniving phase in the cell cycle, microtubules are organized around a central organelle, termed centrosome, that is composed of a pair of centrioles. In most situations, centrosome duplication is critical for the formation of mitotic spindles and normal cell division (2, 3). Removal of centrosomes by micromanipulation techniques results in G1 phase cell cycle arrest and the inability to undergo cytokinesis (4, 5). Among several species of algae and protozoans and in animal epithelial cells and sperm cells, centrosomes are called basal bodies that trigger the formation of flagella and cilia (6).

Studies in erythrocytic form parasites suggest that Plasmodium parasites do not possess the well defined cell cycle events that are present in typical higher eukaryotes. Based on its unique characteristics, cell division in malaria parasites is classified as cryptomitosis (1, 7) where throughout the cell division the nuclear membrane remains intact. Another characteristic
feature in these parasites is the presence of a single centriolar plaque engaged in the pore of the nuclear membrane that gives rise to a half-spindle or hemispindle (8). The centriolar plaque subsequently divides to give rise to two daughter hemispindles that move in opposite directions and form a complete spindle (1).

In recent years, significant progress has been made toward the understanding of the molecules and cellular processes that regulate centrosome duplication in higher eukaryotes. In this context, centrin, a low molecular weight protein that belongs to the EF-hand superfamily of calcium-binding proteins has been found to be associated with centrioles, centrosomes, and mitotic spindle poles (9). In line with its Ca\(^{2+}\) binding characteristics, centrin-based fiber systems demonstrate Ca\(^{2+}\)-induced contractile motion (10, 11). The critical role of centrin in the cell cycle is evident from the studies showing that experimentally induced mutations in the centrin gene in yeast, Chlamydomonas, mice, and humans results in failure of centrosome/basal body duplication and cell cycle arrest (12–15). In trypanosomatid protozoans Leishmania donovani and Trypanosoma brucei, centrins play a critical role in cell division (16–18). In L. donovani, genetic deletion of centrin-1 results in specific failure of basal body duplication, arrested cytokinesis, and a severely impaired growth (16). RNA interference-mediated knockdown in the expression of the centrin-1 gene in T. brucei results in failure of organelle segregation and cytokinesis (19). Furthermore, in human HeLa cells knockdown of centrin-2 by RNA interference resulted in failure of centriole duplication during cell division (14). These results clearly point toward the critical role of centrins in the regulation of the cell cycle probably mediated through its participation in centrosome/basal body duplication.

In this communication, we report the biological characterization of Plasmodium falciparum centrin (PfCEN)\(^2\) molecules, and by means of growth complementation studies in Leishmania parasites, we demonstrate their function in cell division. To this end, we have performed gene isolation, phylogenetic analysis, and determined the stage-specific expression and cellular localization of four centrin molecules in P. falciparum malaria parasites. We also determined the effect of episomal expression of P. falciparum centrin genes in growth-deficient L. donovani parasites that have deletion in their centrin-1 gene.

**EXPERIMENTAL PROCEDURES**

**Parasite**

Synchronized P. falciparum 3D7 parasite cultures were maintained in a temperature cycling incubator with a periodicity of 48 h (20). Synchronously growing parasites were harvested at ring, trophozoite, and schizont stages of development. Gametocyte culture was set up as described previously, and synchronized erythrocytic stages (mixture of ring, trophozoite, and schizont) using High Pure RNA isolation kit (Roche Applied Science). A total of 2 \(\mu\)g of RNA from the blood stage parasites was reverse-transcribed using random hexamers and Superscript II RNase H\(^{-}\) reverse transcriptase (Invitrogen) in a 20-\(\mu\)l volume. 5 \(\mu\)l of the reverse-transcribed product was subjected to PCR using PfCentrin-specific primers. The PCR product was cloned in pET 101/D-TOPO vector and transformed in TOP 10 electrocompetent cells as per the kit’s instruction (Invitrogen). The recombinant plasmids containing the four centrin genes were used to determine the DNA sequence.

**Sequence and Phylogenetic Analysis**

Sequence similarity searches of the protein sequence data bases were performed using the BLASTPGP program and those of the DNA sequence data bases using the TBLASTN and BLASTN programs. The initial differentiation of putative centrin homologs from those of other EF-hand proteins like calmodulin was carried out using reciprocal BLASTPGP searches against individual proteome data bases for each of the species included in our study. This was then followed by rigorous phylogenetic analysis to delineate the different orthologous groups and lineage-specific expansions. The maximum likelihood (ML) tree was derived from a multiple sequence alignment of the centrins selected to include a diverse set of completely sequenced eukaryotic lineages, namely animals, fungi, plants, apicomplexans, ciliates, kinetoplastids, heterolobosans, parasalids, and diplomons. The multiple sequence alignment was constructed using the Kalign program (21). The among-site variation of rates for the alignment was modeled as a distribution with four discrete rate categories (because the alignment length is short), and the positions belonging to each rate category, and the \(\alpha\)-parameters of the distribution were estimated using the TreePuzzle 5.1 program with JTT matrix (22). This was used to infer the ML tree with PROML from the Phylib package, and bootstrap support was estimated using 500 replicates with the PHYML program (23) and RELL-BP (10,000 replicates) with PROML. A parallel neighbor-joining tree was constructed using the Mega4 package (24) by using heterogeneous rates across sites modeled using different \(\alpha\)-parameters. The \(\alpha\)-parameter chosen for the final analysis was the one that reproduced the species tree consistently within different centrin orthologous groups.

\(^2\) The abbreviations used are: PfCEN, P. falciparum centrin; LdCEN, Leishmania donovani centrin; DAPI, 4',6-diamidino-2-phenylindole; EB, endoplasmic reticulum; IEM, immunoelectron microscopy; KO, Gene knockout; MTOC, microtubule organizing center; RAP1, Rhoptry-associated protein 1; WT, wild type; PIPES, piperazine-\(\text{N},\text{N}'\)-bis(2-ethanesulfonic acid); BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HA, hemagglutinin; PBS, phosphate-buffered saline.
**Expression of PfCENs in Escherichia coli**

Four PfCENs were produced as recombinant proteins in BL21 *E. coli*. Following transformation of *E. coli* cells with recombinant plasmids, expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. The histidine-tagged recombinant protein was purified on a Ni²⁺-nitrilotriacetic acid column as per the manufacturer’s instructions (Novagen, Gibbstown, NJ).

**Antibodies**

Antibodies to the four PfCENs were generated by immunizations in mice with recombinant centrins. Six- to 8-week-old female CD1 mice used in immunizations were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in accordance with the guidelines set forth by the National Institutes of Health manual “Guide for the Care and Use of Laboratory Animals.” Mice were immunized (five per group) subcutaneously with 10⁶ of the purified protein in Freund’s complete adjuvant and boosted 3 and 6 weeks later with 10⁵ of protein in Freund’s incomplete adjuvant. Control mice received Freund’s adjuvant only. Serum samples were collected at the time of each immunization and 10 days after the last immunization and stored at −20 °C.

A mouse monoclonal anti-α-tubulin antibody (Sigma) was used to stain the microtubule network (25). A polyclonal rat anti BiP antibody was used as an endoplasmic reticulum (ER) marker (26, 27), and mouse anti-RAP1 monoclonal antibody (7H8/50) was used as a marker for rhotries (28).

**Stage-specific Expression of PF CENs**

**Real Time Quantitative PCR**—Total RNA was isolated from *P. falciparum* synchronized erythrocytic (ring, trophozoite, schizont, and gametocyte) and sporozoite stages using High Pure RNA isolation kit (Roche Applied Science). A total of 2 μg of RNA was reverse-transcribed using random hexamers and Superscript II RNase H⁻ reverse transcriptase (Invitrogen) in a 20-μl volume. Quantitative real time PCR was carried out in triplicate using MyiQ single color real time PCR detection system (Bio-Rad). Gene-specific primers were designed using Beacon Designer4.0 software (supplemental Table S1). Each reaction was optimized to contain equal amounts of cDNA, 100 ng of gene specific primers, and 1 × SYBR Green PCR mix (Bio-Rad). Cycle threshold (CT) values were determined by using iCycler software. Standard curves for each centrin gene were obtained by using different concentrations of wild type *P. falciparum* genomic DNA (10² to 10⁻⁵ ng/μl) as template. The relative genomic equivalence of individual centrin genes was determined by using the respective CT values. The *P. falciparum* fructose-biphosphate aldolase gene was used to normalize the relative genomic equivalence for each centrin gene (29).

**Indirect Immunofluorescence**—Spots of *P. falciparum* sporozoites, erythrocytic and gametocyte stage parasites were made on 12-well glass slides. Parasites were incubated with mouse anti-PfCEN antibody specific to PfCEN1, -2, -3, and -4, and centrin-antibodies complex was detected by incubation with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Slides were mounted using VectaShield mounting medium for fluorescence (Vector Laboratories Inc., Burlingame, CA) and evaluated using an Olympus BX-50 microscope equipped with Olympus DP71 digital camera (Olympus Imaging America Inc., Center Valley, PA). Images were transferred to the PC version of Adobe Photoshop 5.0 for labeling and printing.

For oocyst-stage parasites, midguts were fixed in 4% paraformaldehyde for 2 h at room temperature and washed three times for 10 min each in 1 × PBS. Then the midguts were permeabilized in 5% bovine serum albumin, 0.2% Triton X-100 for 2 h at room temperature. The midguts were incubated with mouse anti-PfCEN antibodies specific for PfCEN1, -2, -3, and -4 in permeabilization buffer for 1 h at room temperature. Samples were washed with 1 × PBS three times for 10 min each. Centrin-antibodies complex was detected by incubation with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 1 h in permeabilization buffer. Midguts were mounted onto glass slides with VectaShield mounting medium for fluorescence (Vector Laboratories) and evaluated using an Olympus BX-50 microscope as described earlier.

For confocal and widefield epifluorescence microscopy, *P. falciparum* asexual blood stage schizonts were fixed in 3% EM grade formaldehyde in PBS, 10 mM PIPES, pH 6.5, for 30 min at room temperature and were allowed to settle onto lysine-coated coverslips. The infected red cells were permeabilized in 0.25% Triton X-100 in PBS for 10 min at room temperature, washed once in PBS for 5 min, and incubated in 3% bovine serum albumin in PBS (blocking solution) for 1 h at room temperature. The primary antibody (PfCEN3 and/or α-tubulin/or BiP/RAP1) was diluted in blocking solution and incubated with the samples for 1 h at room temperature. The samples were washed three times for 5 min in PBS and then incubated in a secondary antibody (Alexa Fluor 488- or Alexa Fluor 568-labeled goat anti-mouse IgG or goat anti-rat IgG (Invitrogen)) diluted in blocking solution for 1 h. For double labeling of PfCEN3 and tubulin, PfCEN3 was labeled with Alexa Fluor 488 goat anti-mouse IgG and tubulin was labeled with the Zenon® Alexa Fluor 680 Mouse IgG1 kit following the manufacturer’s instructions. Samples were washed once in PBS and then stained with 1 μg/ml DAPI in PBS for 10 min. Samples were washed for two additional times in PBS, swirled briefly in distilled water, and then mounted in VectaShield antifade reagent (Vector Laboratories). Confocal images were obtained with a Leica TCS-AOBS/SP2 confocal microscope with a 100×, 1.3 NA, or a 63×, 1.4 NA objective with zoom setting 6–8. Widefield epifluorescence images were obtained with a 100×, 1.3NA objective and cooled CCD camera as described previously (25). Images were adjusted for contrast in Adobe Photoshop 5.5 (Adobe Systems).

**Immunoelectron Microscopy**

Immunoelectron microscopy was carried out on *in vitro* cultured blood stage schizonts of *P. falciparum* and sporozoites isolated from infected mosquito salivary glands using 1:40 anti-PfCEN2 antibody by using a procedure described earlier (30).
Plasmodium falciparum Centrins

Episomal Expression of PfCEN in L. donovani Centrin Knock-out Parasites and Growth Complementation Studies

To determine the functional role of centrin in malaria, PfCENs were transfected in centrin-1 deleted L. donovani null mutants (LdCEN1KO) (16).

P. falciparum centrin genes encoding for PfCEN1, -2, -3, and -4 were PCR-amplified using P. falciparum centrin containing TOPO plasmids as template. The primer sequences and restriction sites used to amplify the recombinant constructs are given in supplemental Table S1. The PCR-amplified centrin genes were initially cloned at the T/A cloning site of pCRII-TOPO cloning vector. The fidelity of the cloned sequences was verified by nucleotide sequencing. The centrin gene inserts were further cloned in BamHI restriction sites of the Leishmania expression plasmid PHLEO (31). The resultant recombinant plasmids, pXG-PHLEO-PfCEN1, pXG-PHLEO-PfCEN2, pXG-PHLEO-PfCEN3, and pXG-PHLEO-PfCEN4 were transfected individually into the LdCEN1KO L. donovani promastigotes by electroporation of recombinant plasmid DNA. The detailed procedure for episomal transfection in L. donovani has been described previously (16). Transfected promastigotes were initially selected in the presence of 10 μg/ml phleomycin (Sigma) and then after each culture cycle were grown in gradually increasing concentrations of phleomycin that finally reached 150 μg/ml. Finally, transfected Leishmania growing in the presence of 150 μg/ml of drug were used in subsequent experiments.

The episomal expression of the hemagglutinin-tagged PfCENs in recombinant Leishmania lines was determined in an ECL-based Western blot. Briefly, 45 × 10^5 PfCENs-transfected amastigotes were resolved on 4–12% BisTris NuPAGE gel and electroblotted on to polyvinylidene difluoride membrane. The membrane was probed with anti-HA tag (1:1000) and anti-α-tubulin (1:8000) antibodies, and blots were developed using a chemiluminescence-linked Western blot kit (Western Light Tropix, Bedford, MA). To study whether transfection of P. falciparum centrins in the LdCEN1KO parasite line was able to reconstitute the loss of Leishmania centrin function, recombinant centrin-expressing promastigotes were moved into medium for axenic amastigote growth with phleomycin (150 μg/ml), and their growth rate was monitored along with the LdCEN1KO parasites and wild type (WT) parasites as controls.

RESULTS AND DISCUSSION

Identification and Cloning of PfCEN Genes—By sequence similarity searches using the BLASTPGP program, we identified the sequences of four P. falciparum centrin homologs. These molecules are located on chromosomes 1 (NP_703272), 10 (NP_700744), 11 (NP_700931), and 14 (NP_702332) and were annotated as PfCEN1 (chromosome 1), PfCEN2 (chromosome 14), PfCEN3 (chromosome 10), and PfCEN4 (chromosome 11). Further sequence searches in PlasmoDB has led us to identify two centrin homologs in Plasmodium vivax (Pv081420 and Pv111135), three in Plasmodium yoelii (XP_727858, XP_726416, ad XP_723980), and four each in Plasmodium berghei (PB000338.02.0, PB000363.00.0, PB000584.00.0, and PB001391.02.0) and Plasmodium knowlesi (PKH_020620, PKH_061200, PKH_090270, and PKH_125980).

By using gene-specific primers (supplemental Table S1), we amplified all four PfCEN genes from a cDNA library prepared from blood stage parasites. We found that for the centrins PfCEN3 and PfCEN4, the amplified gene products were smaller than their sizes as predicted in PlasmoDB. PfCEN3 amplified as a 540-bp gene instead of the expected 750 bp, and PfCEN4 amplified a 516-bp product instead of 618 bp. Amplification of PfCEN1 and PfCEN2 genes results in fragments of 507 bp each that matched their expected size (supplemental Fig. S1). We cloned these amplified products in pET 101/D-TOPO plasmid and transformed into TOP 10 cells and determined their nucleotide sequence. PfCEN1 and PfCEN2 sequences correspond to the original PlasmoDB base data sequences. For the PfCEN3 gene, our nucleotide sequence matched with the sequence predicted by FullPhat (chr10.phat_285) instead of the annotation presented by TIGR (PF10_0271) that shows the presence of three introns instead of the four introns present in our PfCEN3. Similarly, for PfCEN4 the gene annotation by TIGR designated as PF11_0066 shows the presence of one intron, whereas we find two introns as predicted by Genefinder chr11.gen_32.

All four PfCEN genes have multiple exons as follows: six in PfCEN1, five in PfCEN3, and three each in PfCEN2 and -4 (supplemental Fig. S2), which is comparable with the multiple introns found in the centrin genes of other unicellular eukaryotes, fungi, plants, and animals (32, 33). The P. falciparum centrin genes PfCEN1, -2, -3 and -4 encode proteins of length 168, 168, 179, and 171 amino acids, respectively.

Sequence and Phylogenetic Analysis of Centrins—All the four PfCENs fit the expected pattern described for centrin molecules from other organisms in possessing four conserved EF-hand domains that are organized in the form of a pair of two EF-hand units connected by a flexible linker (34). The PfCENs display extensive sequence variability in the N-terminal regions (i.e. upstream of the first EF hand) and in the linker regions (Fig. 1). These N-terminal extensions in the PfCENs (~20–30 amino acids long) contain predicted basic nuclear localization signals that direct the protein to the nucleus (35–37). In an EF-hand loop, the calcium ion is coordinated in a pentagonal bipyramidal configuration; six residues involved in the binding are in positions 1, 3, 5, 7, 9 and 12. In this arrangement, the invariant Glu or Asp at position 12 provides the ligand to chelate Ca^2+ (38–40). We aligned the four central calcium-binding loops of the PfCENs with the consensus amino acids to predict the functional calcium-binding domain (supplemental Fig. S3). Analysis showed that for PfCEN1 and PfCEN3, any of EF-hands 1, 3, or 4 could be functional, whereas EF-hand 2 did not match the consensus. For PfCEN4, the third EF-hand could be functional; the remaining three EF-hands did not fit the consensus sequence (supplemental Fig. S3). We determined if PfCENs indeed possessed the Ca^2+-binding property. To accomplish this, all four PfCENs were produced as histidine-tagged recombinant proteins in E. coli. SDS-PAGE showed that following the purification on a Ni^2+-nitrilotriacetic acid column, recombinant PfCEN1, -2, -3, and -4 were purified to relative homo-
that the PfCENs are indeed the members of the Ca\(^{2+}\) (data not shown). These observations support the contention that the parabasalid etoplastids and at least 12 in the early branching eukaryote, are multiple centrins, at least 4 in most alveolates and kinetoplastids. This suggests that at least some centrin functions are likely to have been maintained unchanged throughout eukaryotic evolution. The early branching eukaryote, the diplomonad Giardia lamblia, possesses two bona fide centrins, suggesting that there could have been at least two distinct centrins in the common ancestor of extant eukaryotes. In several unicellular as well as multicellular eukaryotes, there are multiple centrins, at least 4 in most alveolates and kinetoplastids and at least 12 in the early branching eukaryote, the parabasalid Trichomonas vaginalis indicating multiple lineage-specific duplications. Given that unicellular eukaryotes with extensively developed flagellar or ciliary systems (e.g. alveolates, kinetoplastids, and parabasalids) typically have a greater number of centrins than other unicellular organisms lacking these organelles (e.g. yeasts), this suggests a potential functional sub-specialization of the expanded centrins beyond the basic role in cell division.

To further understand the evolutionary affinities of the Plasmodium centrins, we subjected the centrins to a phylogenetic analysis using neighbor-joining and maximum likelihood methods. An important caveat to note is that the centrins are short proteins, and the different paralogs are evolving at different rates. Hence, it was critical to obtain an appropriate estimate of the heterogeneity in rates to obtain meaningful phylogenetic trees in both methods and to estimate support for nodes arising through a duplication in their common ancestor. Plasmodium possesses all four centrins of the ancestral alveolate, whereas other genera (Theileria, Toxoplasma, and Cryptosporidium) show loss of one of the centrins (Fig. 2). Toxoplasma and Cryptosporidium contain two additional fast-evolving centrins (TgCen04 and cg08_500) that appear to have emerged via a duplication in the ancestral alveolate clade defined by PfCEN2 and TgCen02. This has resulted in Toxoplasma and Cryptosporidium having the same count of centrins as Plasmodium, even though both these lineages have lost one of the ancestral alveolate-specific centrins (Fig. 2; the evolutionary concordance between the different eukaryotic centrins is shown in Table 1). The monophyly of each of the four alveolate centrin clades is well supported (>80% in both direct ML and RELL-BP bootstrap estimates) taking into account the short length of the input alignment for tree construction. The kinetoplastids (Trypanosoma and Leishmania) also possess one representative of both the principal centrin clades traceable to the last eukaryotic common ancestor (Fig. 2). However, they possess at least two additional lineage-specific duplications that have spawned distinctive kinetoplastid-specific centrins. Interestingly, the basal eukaryote T. vaginalis shows an extraordinary lineage-specific expansion in the clade corresponding to the mammalian centrin-1/2/4 to give rise to at least seven distinct paralogs.

Given the high degree of similarity between centrins from diverse eukaryotes, we investigated the cross-species antigenic homology of PfCENs using Western blot analysis. Recombinant PfCENs were subjected to SDS-PAGE separation and then transferred on a polyvinylidene difluoride membrane and then reacted with anti-centrin antibodies. As shown in supplemental Fig. S5, recombinant PfCEN3 cross-reacts widely with the antibodies against Chlamydomonas reinhardtii centrin, human centrins (HsCen2p and HsCen3p), and L. donovani centrin1. In addition L. donovani centrin antibody also recognizes PfCEN2 and -4. The cross-reaction of PfCENs with centrins from other species shows that in addition to sequence and domain homology, at protein level they also shared cross-reacting B-cell epitopes throughout eukaryotic phylogeny.

Stage-specific Expression and Cellular Localization of PfCENs—The stage-specific expression of PfCENs was determined at both transcriptional and protein level in P. falciparum using both traditional bootstrap and RELL-BP methods (10,000 replicates). The consensus phylogenetic tree from the two methods (Fig. 2) supports the contention that the ancestral eukaryote possessed at least two distinct centrins. The first of these corresponds to Centrin1/2/4 of mammals and the second of these to the mammalian Centrin3 and CDC31 of yeast. Alveolates, including Plasmodium, possess the orthologous representatives of both these centrin lineages (Fig. 2). The alveolates also possess two additional centrins that appear to have}

**FIGURE 1.** Amino acid sequence alignment of P. falciparum centrin-1, -2, -3, and -4. Identical amino acids are highlighted in black; residues showing ≥80% similarity among the four Plasmodium centrins are shaded in gray. The predicted four EF-hand domains are boxed. The central calcium-binding loop is further boxed. The α-helical domains are marked by dotted line.
sporozoite, asexual blood forms of rings, trophozoites, and schizonts, and in late gametocytes. Also, protein expression was detected in the *P. falciparum* oocysts developing in mosquito mid-gut. Transcription of PfCEN-specific RNA was determined by quantitative real time PCR and demonstration of protein expression by immunofluorescence analysis. Antibodies to PfCEN1, -2, -3, and -4 were generated by immunizations in outbred CD1 mice with *E. coli*-produced recombinant PfCENs delivered in Freund's adjuvant.

Quantitative real time PCR shows that in relative terms the expression of centrins is the highest in gametocyte stage, followed by blood stage parasites, and the lowest in sporozoite stage of parasite cycle (Fig. 3). This observation is in general agreement with the stage-specific expression of PfCENs as determined by microarrays (41) and is available in *P. falciparum* transcriptome data base in PlasmoDB. In sporozoite stage, *PfCEN2* and *PfCEN3* have a relatively low level of expression, whereas *PfCEN1* and *PfCEN4* had barely measurable expression. In blood stages, only *PfCEN3* had measurable expression in the ring form; the other three centrins were essentially absent. In trophozoites, the highest expression was detected for *PfCEN3*, and moderate levels for *PfCEN1* and *PfCEN4*, whereas *PfCEN2* was found to be absent. Surprisingly, in mature schizonts, only *PfCEN2* and *PfCEN3* had high level of expression, whereas *PfCEN1* and *PfCEN4* were expressed at a low level. Thus, a great degree of variation in centrin expression existed during the parasite development inside erythrocytes that supports the view that via transcriptional regulation individual centrins might play a distinct role during the schizogony and the subsequent maturation of merozoites. At gametocyte stage, *PfCEN1* and *PfCEN2* had the highest expression, but *PfCEN3* and *PfCEN4* were also expressed at relatively high levels (Fig. 3).

We next determined the expression and cellular localization of *PfCEN1*, -2, -3, and -4 at schizont, gametocyte, and oocyst stages of malaria parasites by IIF using specific antibodies. Results show that all four anti-centrin antibodies reacted with the schizont stage of the parasite (Fig. 4A). A strong fluorescence signal was observed for *PfCEN2* and *PfCEN3* and a
evolutionary concordance of centrin paralogs from a representative set of eukaryotic lineages

| Organism/lineage | Centrin-1 clade | Centrin-3 clade | Alveolate-type centrins | Kinetoplastid-type centrins | Other unique centrins |
|------------------|-----------------|-----------------|-------------------------|----------------------------|----------------------|
| Mammals          | HsCen1, HsCen2, HsCen4 | HsCen3 | | | |
| Yeast            | CDC31p ECU07, 1230 | | | | |
| Encephalitozoon  | (19074546) | | | | |
| Plants           | ATCEN2 (15229732), ATAG37010 (79326379) | | | | |
| Chlorophyte algae | VLF2 (Chlamydomonas) | | | | |
| Plasmodium       | PfCEN1 (MAL1P1.61) | PfCEN3 (PF10_0271) | PfCEN2 (PF14_0443) | PfCEN4 (PF11_0066) | |
| Theileria a      | Tp03_0058, TA02500 | Tp01_0272, TA19510 | TA17720, TP03_0609 | | |
| Toxoplasma       | TgCEN01 | TgCEN03 | TgCEN02, TgCEN04 | TgCEN01 | |
| Cryptosporidium  | | cgd5, 60 (66357586) | cgd8, 1280, cgd8, 500 | cgd8, 4120 | |
| Ciliates (Tetrahymena) | THERM_00384910 (89301059) | THERM_00523060 (89286251) | THERM_00194330 (89298953) | | |
| Kinetoplastids b | LmjF07.0710 (68124271), TbCent01 (70802975) | Tb927.4.2260 (70800909), LmjF4.2390 (68124911) | | | |
| Naegleria        | Ngru1000004390 | Ngru1000001693 | | | |
| Trichomonas      | TVAG_017360, TVAG_238650, TVAG_404740, TVAG_026750, TVAG_029880, TVAG_311330, TVAG_238650, TVAG_357970 | TVAG_252700, TVAG_335140, TVAG_376480 | | | |
| Giardia          | GLP_487_22250_22735 (29251100) | GLP_158_56914_57444 (29250378) | | | |

a The representatives from both species of Theileria, T. parva and T. annulata, are provided in the Theileria row.
b The representatives from Trypanosoma brucei and Leishmania are provided in the kinetoplastid row.
c gi numbers are provided in certain cases to allow identification of the specified sequence.

Weaker signal for PfCEN1 and -4. Anti-centrin antibodies to PfCEN1, -2, -3, and -4 also reacted with the gametocyte stage of the parasite. Again the intensity of reaction was stronger for PfCEN2 and -3 (Fig. 4B). During the oocyst stage, IIF reactivity was observed with PfCEN1 and -3, whereas no fluorescence signal was obtained for PfCEN2 and PfCEN4 (Fig. 4C). Together, results from transcription analysis and protein expression analysis demonstrated that in P. falciparum parasites PfCENs are differentially expressed during different stages of the life cycle, which is consistent with the possibility that the centrins perform distinct roles in the cell cycle regulation and development.

In higher eukaryotes, tissue-specific functional roles for different centrins have been demonstrated. For example, in mammals four centrin proteins have been identified so far. Centrin-1 is mostly expressed in retina and male germ cells and localizes at the centrosome and the basal bodies (42, 43). Centrin-2 and -3 are ubiquitously expressed and localize in the distal lumen of centrioles and in the pericentriolar bud (44–46). Centrin-4 is expressed exclusively in brain, kidney, lung, and ovary and localizes at the basal bodies (32). Based on localization centrins can be categorized in two subgroups, ones that are ubiquitously expressed (centrin-2 and -3) or ones that are more specifically expressed, i.e. ciliary centrins (centrin-1 and -4). Mammalian centrin-1 and -2 belong to the same centrin clade as that from the green alga C. reinhardtii, which forms Ca2+-dependent contractile fibers required for proper segregation of flagellar apparatus during cell division (11, 47). Mammalian centrin-3 (ortholog of Saccharomyces cerevisiae, Cdc31p) is involved in spindle pole body duplication (12, 48). Centrin-4 is more specifically involved in basal body assembly or in a step of ciliogenesis (32). Localization of centrins at the centrosomes and basal bodies has led to many hypotheses of the function of centrins. When the cells are in interphase stage, the centrosome functions as the major microtubule organizing center (MTOC) determining the number and the polarity of cytoplasmic microtubules. New polymerizing microtubules are nucleated in the pericentriolar matrix of the mitotic spindle. In general terms it appears that centrin-3 participates in centrosome reproduction and duplication (50) and centrin-1, -2, and -4 may be involved in centrosome separation preceding centrosome duplication (51).

We also wanted to know the site of centrin expression in P. falciparum sporozoites and blood form schizonts at the subcel-
lular level. This was accomplished by performing immunoelectron microscopy (IEM) and confocal microscopy using centrin-specific antibody. In IEM, sections of sporozoites and asexual blood stage schizonts were incubated with anti-\(\text{PfCEN2}\) antibody. In \textit{P. falciparum} sporozoites, IEM localization of \(\text{PfCEN2}\) was observed as multiple dots in an area surrounding the nucleus suggesting a possible association with the centrosome (Fig. 5A). Similarly, in blood form schizonts, immunogold particles were seen to be associated with an area in proximity to the nucleus (Fig. 5B). Reactivity close to the nucleus area is observed in the form of two discrete dots that gives an appearance of its association with the centrosome. This observation is further substantiated in confocal and widefield epifluorescence microscopy studies where \textit{P. falciparum} schizonts were either double stained with anti-\(\text{PfCEN3}\) (Alexa Fluor 488 label) and/or anti-\(\alpha\)-tubulin (direct Alexa Fluor 680 label), anti-BiP antibodies, or stained with these antibodies separately. In confocal studies, when stained with \(\text{PfCEN3}\) antibody alone, centrin reactivity appears as a pattern of paired spots that are organized around DAPI-positive nuclei. These spots are either superimposed with the nucleus (white arrow) or appear in its juxtaposition (white arrowhead) (Fig. 6A). Centrioles are known to be embedded in the nuclear membrane, not buried in its central DNA mass, and centrosome elements extend to the cytoplasmic face of the nuclear membrane (52). A careful three-dimensional visualization of the images confirmed that \(\text{PfCEN3}\) reactivity is localized on the “outer” side and not “inside” of the central mass of the DAPI-stained DNA. Further evidence of the centrosomal association of malarial centrin is provided by the fact that the reactivity pattern of \(\text{PfCEN3}\) antibody closely resembles that observed with \textit{C. reinhardtii} 20H5 monoclonal antibody (Fig. 6B), which also cross-reacts with \textit{PfCEN2} and \textit{PfCEN3}, a well characterized marker for centrosome-associated centrin (44, 53). \(\text{PfCEN3}\) reactivity to the outer side of the nuclei suggested localization with centrosomes in the nuclear membrane. Because the parasite nuclear membrane is continuous with the ER surrounding the nucleus, the ER is considered as a visual marker to identify objects embedded in the nuclear membrane. To confirm that \(\text{PfCEN3}\) localizes to centrosomes in the nuclear membrane, parasite ER/nuclear membrane was stained with anti-BiP antibody, which labels an ER resident protein. Wide field and confocal microscopy demonstrate that \(\text{PfCEN3}\) localizes to the outer edge of nuclear DNA and that it is visible in the same focal plane as the ER/nuclear membrane (Fig. 6C). This localization is consistent with the earlier knowledge that centrosomes are embedded in nuclear pores.

Functional eukaryotic centrosomes serve as MTOCs for mitotic spindle. Previous work has shown that during “cryptomitosis” in \textit{Plasmodium} parasites, the membrane-bound centrosomes are located at the poles of the intranuclear mitotic
spindle. To visualize mitotic spindles and confirm their location within nuclei, *P. falciparum* schizonts were stained with anti-α-tubulin antibody and anti-BiP antibody to outline the nuclear membrane as described above. Wide field and three-dimensional confocal microscopy show that the prominent tubulin structures are enclosed within the nuclear membrane and that these microtubules extend into the nuclear DNA (Fig. 6D). These results are in agreement with previous studies (7, 8, 52), and it is reasonable to infer that the anti-α-tubulin reactivity inside the parasite nucleus indeed labels the mitotic spindle.

To further ascertain the localization of PfCEN3 in *P. falciparum* schizonts, synchronized late *P. falciparum* schizonts were subjected to double staining with anti-PfCEN3 and anti-α-tubulin antibodies. As observed in Fig. 6E (panel 4), the centrin antibody (green) reacts in close proximity to DAPI stained nuclei (blue), and α-tubulin (red) antibody reacts with the microtubule network. Centrin dots localize to the ends of prominent tubulin structures in the parasite nuclei as expected for centrosomal proteins. The presence of centrin dots as paired structures that are spaced at various distances from each other suggests the centrin association to the centrosomes during different stages of mitotic division and centrosomal segregation (Fig. 6E, panels i–iii). It appears that with the completion of schizogony, centrin realigns as dots alongside the nucleus (Fig. 6E, panels iv–vi). Taken together, based on the reactivity observed in widefield and confocal microscopy, our results strongly suggest the centrosomal association of PfCEN3 during different phases of cell division in malaria parasites.

In vertebrates, centrosomes are nonmembranous organelles usually located at the center of the cell in close proximity to the nucleus and are predominantly composed of two components, centrioles and pericentriolar material. In motile cells that possess cilia and flagella, centrioles are replaced with basal bodies. In most cells, centrosomes function as the major microtubule organizing center with rapidly growing microtubule ends lying in the cytoplasm and their slow growing ends being attached to the centrosome. However, in members of the phylum Apicomplexa that includes the genus *Plasmodium*, three distinct organelles serve as MTOCs, the polar rings, spindle pole of centrins in *Plasmodium* MTOCs and the role of individual centrins in different stages of the parasite life cycle.

A unique characteristic of apicomplexans is the presence of a set of organelles at their anterior end called apical complex (55). These organelles include the rhoptries, micronemes, and dense granules (54, 56, 57). The contents of these organelles are essential for parasite invasion into host cells and their intracellular replication, survival, and further transmission. For example, products of rhoptries and micronemes participate in the initial contact of the parasite with the host cell, its subsequent invasion, and the formation of parasitophorous vacuole (58, 59). Previous studies have shown that the apical ends of merozoite buds and their associated organelles are initiated and develop close to the cytoplasmic side of centrosomes/spindle pole bodies (52). From these observations, it has been postulated that centrosomes control the site of bud formation and thereby regulate the number of daughter cells at the conclusion of schizogony (60). Centrosomes may function to spatially organize the developing apical organelles in several modes that are not mutually exclusive. Centrosomes may associate directly with organelles during division as shown for Golgi and apicoplasts in *Toxoplasma* or centrosomes may direct the localized biogenesis of some organelles. It has been shown that the ER membrane immediately adjacent to the centrosomal pore is a specialized exit site of the secretory pathway, and this membrane is thought to produce the precursors for the Golgi and other apical organelles such as rhoptries (52, 60).

To view the relative position of PfCEN3 and developing rhoptries, schizonts were stained with anti-PfCEN3 antibody and anti-RAP1 antibody (Fig. 7). Similar to previous studies, we find that the rhoptries stained by anti-RAP1 antibody (red) are arranged in pairs around the periphery of the schizont (Fig. 7). Anti-PfCEN3 staining (green) does not colocalize with anti-RAP1 staining, suggesting that the cytoplasmic portion of PfCEN3 does not directly associate with rhoptries. Although these two markers do not colocalize, a close examination of antibody-reactive spots in confocal microscopy suggests that in most instances the spatial relationship between PfCEN3 and

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**FIGURE 5.** Immunoelectron micrograph showing reactivity with the *P. falciparum* centrin-2 antibody. The gold particles associated with the nucleus of *P. falciparum* sporozoite stage parasite (A) and nucleus of asexual blood stage schizonts (B). *N*, nucleus; *PVM*, parasitophorous vacuole membrane; *RBC*, red blood cell.
**FIGURE 6.** Immunolocalization of *P. falciparum* centrin-3 by confocal and widefield epifluorescence microscopy. For confocal microscopy, *P. falciparum* asexual stage schizonts were stained with mouse anti-PfCEN3 antibody (A) and Cr20H5 monoclonal antibody (B). 1st panel depicts the differential interference contrast (DIC) image; 2nd panel depicts DAPI-stained nucleus (blue); the centrin reactivity is shown in the 3rd panel (green); and the 4th panel represents the merging of blue and green channels. Centrin reactivity superimposed on the nucleus is shown with an arrow; and the centrin reactivity juxtaposed to nucleus is shown with an arrowhead. All images are projected confocal z-series processed using deconvolution software. Scale bar, 2 μm.

**FIGURE 7.** Developing rhoptry pairs are arranged in a symmetrical pattern around PfCEN3 centrosomes. Confocal image of a schizont stage parasite. Nuclei are stained with DAPI (blue), anti-PfCEN3 antibody is labeled green, and anti-RAP1 antibody is labeled red to stain rhoptries. Rhoptries (red) are arranged in pairs around the peripheral cytoplasm of the developing schizont. (RAP1, arrows). PfCEN3-positive centrosomes (green) are located between each rhoptry pair (Merge, arrows).

RAP1 is not random. We find that the PfCEN3 spots localize to the space at the center of each rhoptry pair (Fig. 7), and this pattern is consistent with an arrangement where rhoptries are seen developing close to the cytoplasmic site of centrosomes (52).

In a recent study, MudPIT analysis of *Toxoplasma gondii* apical complex organelles revealed the expression of two centrin proteins, TgCentrin2 and TgCentrin3 (61). In replicating *T. gondii* parasites, *de novo* construction of daughter conoids (rhoptry scaffold) and apical complex is considered the first sign of daughter cell formation (61). In transgenic *T. gondii* lines, expression of EGF-TgCentrin2 was found to be localized at the extreme apical end (confined to the preconoidal rings) and thus suggested a possible role of apical

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complex-associated centrin in the formation and passage of apical complex organelles to daughter cells in apicomplexan parasites (61). Analogous to T. gondii, in malaria parasites formation of rhoptries proceeds along with cell division (52). The symmetrical presence of PfCEN3 at a space that lies at the center of each rhoptry pair (Fig. 7) raises the possibility of an association between malarial centrin with rhoptries. We also find that in IEM following reactivity with PfCEN2 antibodies, gold particles were observed in areas on or around rhoptries (data not shown). It has been postulated that the ancestors of modern apicomplexans adapted to their obligate intracellular parasitic lifestyle in multiple ways, including the loss of flagella (except in male gametes) (62, 63) and development of a closed conoid. The phylogenetic and morphological data points to its origin from an open-sided conoid as seen in colpodellids and perkinsids (64). The colpodellids and Perkinsids have two flagella and an apical complex similar to apicomplexans having open-sided conoid with associated rhoptries. In flagellated cells, centrin is known to be associated with basal body (flagellar roots) (10). Given that the PfCEN2 protein belongs to one of the two centrin clades unique to alveolates, it is conceivable that it was associated with the development of the cytoskeleton of extrasomes, which are uniquely alveolate organelles (e.g. rhoptries and trichocysts). It is also possible that the apicomplexan parasite might have lost its flagellar apparatus along with the development of a closed conoid. Hence, in this context the localization of PfCEN2, TgCentrin2, and TgCentrin3 alongside the rhoptries could also mark the lost or rudimentary flagellar scaffold of these organisms. Consistent with this, it has also been suggested that in malaria parasite centroles are maintained throughout the asexual life cycle to serve as a template for construction of a basal body that forms flagellar axoneme in male gametes (54).

Episomal Expression of PfCENs in Centrin-deficient L. donovani and Determining Their Biological Function by Growth Complementation Studies—In eukaryotes, centrins are primarily shown to be involved in the process of cell division. To determine whether PfCENs played a direct role in cell division, we expressed malarial centrin genes by genetic transfection in growth-deficient L. donovani (LdCEN1KO) parasites generated in our laboratory by a deletion in the centrin-1 gene by a double recombination event (16). At amino acid level, LdCEN1 shares 46% homology with PfCEN1, 43% with PfCEN2, 41% with PfCEN3, and 29% with PfCEN4.

Centrin null mutant Leishmania promastigotes exhibited normal basal body duplication, and their growth rate was comparable with wild type parasites. However, after transition to axenic amastigote stage, LdCEN1KO parasites had a defect in basal body duplication and a failure in cytokinesis resulting in the creation of multinucleated “large” cells that had severely impaired growth (16). Expression of PfCENs into Leishmania parasites was accomplished by the construction of recombinant PfCEN-pXG-PHLEO plasmids that contained the full-length PfCEN genes in a Leishmania expression plasmid. Recombinant plasmids were independently transfected into the centrin disrupted L. donovani promastigotes, and four genetically transformed Leishmania cell lines were transferred to axenic amastigote cultures conditions in the presence of antibiotic phleomycin. Western blot analysis of the recombinant Leishmania lines confirmed the episomal expression of the hemagglutinin-tagged four PfCENs as determined by reactivity with anti-HA tag antibodies (Fig. 8A). However, transfection-mediated, episomal
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TABLE 2

R^2 values for the fit of the linear and second degree polynomial models and estimates of second degree polynomial regression parameters and their 95% confidence intervals.

For description of the linear and second degree polynomial models and estimates of second degree polynomial regression parameters, see Ref. 65.

| Centrin | Linear model | Second degree polynomial | Parameter | Estimate | 95% confidence interval |
|---------|--------------|--------------------------|-----------|----------|-------------------------|
| LdWT    | 0.77         | 0.92                     | Intercept | 1.86     | 1.52, 2.20              |
|         |              |                          | Time      | 0.048    | 0.040, 0.056            |
|         |              |                          | Time^2    | -6.0 x 10^-4 | -8.0 x 10^-4, -4.0 x 10^-4 |
| LdCEN1KO| 0.73         | 0.94                     | Intercept | 0.262    | 0.222, 0.266            |
|         |              |                          | Time      | 0.003    | 0.0026, 0.0034          |
|         |              |                          | Time^2    | -4.45 x 10^-5 | -3.4 x 10^-5, 4.6 x 10^-5 |
| PfcEN1KO| 0.84         | 0.86                     | Intercept | 0.186    | -0.088, 0.460           |
|         |              |                          | Time      | 0.015    | 0.012, 0.018            |
|         |              |                          | Time^2    | -5.21 x 10^-5 | -1.3 x 10^-5, 3.0 x 10^-5 |
| PfcEN2KO| 0.80         | 0.87                     | Intercept | 0.264    | 0.180, 0.340            |
|         |              |                          | Time      | 0.005    | 0.004, 0.006            |
|         |              |                          | Time^2    | -3.9 x 10^-5 | -5.0 x 10^-5, -3.0 x 10^-5 |
| PfcEN3KO| 0.70         | 0.77                     | Intercept | 0.232    | 0.134, 0.330            |
|         |              |                          | Time      | 0.004    | 0.0028, 0.0052          |
|         |              |                          | Time^2    | -3.59 x 10^-5 | -8.0 x 10^-6, -8.0 x 10^-5 |
| PfcEN4KO| 0.70         | 0.84                     | Intercept | 0.283    | 0.243, 0.323            |
|         |              |                          | Time      | 0.004    | 0.0032, 0.0048          |
|         |              |                          | Time^2    | -4.58 x 10^-5 | -7.0 x 10^-5, -3.0 x 10^-5 |

expression in Leishmania parasites resulted in the production of varying amounts of PfCENs. The reason for the differential levels of expression of PfCENs in Leishmania parasites is not clear. Reactivity with an anti-α-tubulin antibody used as a loading control suggested only a minimal variation in the number of transfected Leishmania parasites used to determine the recombinant expression (Fig. 8A), and therefore, cell counts are not accountable for the observed differences in PfCEN expression. It is important to note that in Leishmania parasites the expression of the four PfCENs is under a uniform transcriptional control in pXG-PHLEO plasmid, and the protein expression was measured using an antibody that detected common B-cell epitope(s) on the influenza-hemagglutinin molecule.

A large sub-set of the pXG-PHLEO-PfCEN1 transfected parasites differentiated into a normal cell phenotype and attained a growth pattern that was significantly distinct from the other three PfCEN transfected parasite lines (Fig. 8B). Transfection of PfCEN1 restored the growth of LdCEN1KO Leishmania parasites by 37%; the other three PfCENs had no effect on the growth of LdCEN1KO null mutants (Fig. 8B). To examine the temporal changes in the reconstitution of L. donovani centrins (as measured by cells × 10^7/ml, see Table 2), second degree polynomial regression model was fitted to the data using the three replicate samples of each of the four PfCENs. The JMP version 6 software was used for the analysis of the regression models. The estimates of the regression coefficients and their 95% confidence intervals for the second degree polynomial regression model are given in Table 2 (65). The estimates of the Time and Time^2 coefficients for the WT and LdPfCEN1 centrins are higher than those of the other centrins. Furthermore, there is no overlap of the 95% confidence intervals for Time and Time^2 regression coefficients of WT and LdPfCEN1 with those of the other three centrins. This indicates that the growth curves of these two parasite lines are significantly different from the other Leishmania lines tested. The results in Table 2 also show that all regression coefficients and their 95% confidence intervals for LdCEN1KO, LdPfCEN2, LdPfCEN3, and LdPfCEN4 centrins are very similar, and there appears to be no statistically significant differences between them. Whether the amount of PfCEN protein expressed in Leishmania parasites influenced its ability to perform the growth complementation function is not known.

In concordance with the growth reconstitution effect, transfection with PfCEN1 also led to a 40% reduction in the number of giant multinucleated cells (Fig. 8C). In comparison, LdCEN1KO amastigotes transfected with the other three centrins, PfCEN2, PfCEN3, and PfCEN4, showed no reduction in the number of multinucleated cells (Fig. 8C). Thus the expression of recombinant PfCEN1 in LdCEN1KO Leishmania parasites was able in part to overcome the growth defect seen in LdCEN1 axenic amastigotes, whereas the other P. falciparum centrins could not rescue the growth rate or multinucleated cell phenotype.

Our results clearly demonstrate that in Leishmania parasites, PfCEN1 supplemented the loss of function caused by the deletion of LdCEN1 genes and thus performed a function that is comparable with a molecule involved in the process of cell division. To our knowledge, this is the first Plasmodium molecule that can be directly attributed to be involved in the process of cell division. Further understanding of the role of individual centrins in the regulation of cell division and development in P. falciparum parasites awaits genetic transfection-mediated cen- trin disruption studies. Such experiments are currently in progress in our laboratory.

Identification and biological characterization of P. falcipa- rum molecules involved in cell division will provide us insight in the obscure area of the cell cycle machinery and growth regulation in malaria parasites. Furthermore, systematic disruption in P. falciparum cen trin genes might allow the construction of growth-deficient malaria parasites for vaccine use. Because expression of centrins is detected during the sporozoite, blood form, gametocyte, and mosquito stages of parasite development, deletion in blood form parasite genome could result in attenuation in sporozoite or blood form parasites. It is also possible that centrin deletion could result in transmission-deficient strains of P. falciparum parasites.
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