Ancient *Leishmania* coronin (CRN12) is involved in microtubule remodeling during cytokinesis

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Accepted 10 February 2009
Journal of Cell Science 122, 1691-1699 Published by The Company of Biologists 2009
doi:10.1242/jcs.044651

Summary

In general, coronins play an important role in actin-based processes, and are expressed in a variety of eukaryotic cells, including *Leishmania*. Here, we show that *Leishmania* coronin preferentially distributes to the distal tip during cytokinesis, and interacts with microtubules through a microtubule-based motor, kinesin K39. We further show that reduction in coronin levels by 40-50% in heterozygous coronin mutants results in generation of bipolar cells (25-30%), specifically in the log phase, owing to unregulated growth of the corset microtubules. Further analysis of bipolar cells revealed that the main cause of generation of bipolar cell morphology is the intrusion of the persistently growing corset microtubules into the other daughter cell corset from the opposite direction. This defect in cytokinesis, however, disappears upon episomal gene complementation. Additionally, our attempts to prepare homozygous mutants were unsuccessful, as only the aneuploid cells survive the selection process. These results indicate that coronin regulates microtubule remodeling during *Leishmania* cytokinesis and is essentially required for survival of these parasites in culture.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/10/1691/DC1

Key words: Trypanosomatids, Cytokinesis, Coronin, Microtubule remodeling

Introduction

Coronins represent an evolutionarily conserved family of WD-repeat actin-binding proteins and are widely expressed among eukaryotes from yeast to humans (Xavier et al., 2008; Morgan and Fernandez, 2008). Whereas higher eukaryotes express variable number of coronin isotypes (Uetrecht and Bear, 2006), only unique coronins are expressed in the lower eukaryotic organisms (Xavier et al., 2008). These proteins localize mainly at the sites where active actin-network remodeling takes place, such as leading edge, phagocytic-cup and immunological synapse (Gandhi and Goode, 2008; de Hostos, 2008; Nal et al., 2004). Using various genetic approaches, coronins have been shown to affect several actin-based cellular functions, such as cell locomotion, phagocytosis, macropinocytosis, cytokinesis and phagosome formation (Nagasaki et al., 2001; Rappleye et al., 1999; Bharathi et al., 2004; Yan et al., 2005). Apart from actin, coronins have also been reported to associate with microtubules. The purified coronin proteins from *Saccharomyces cerevisiae* (CRN11) and *Drosophila melanogaster* (CRN7) have been reported to bind microtubules and to crosslink microtubules and actin filaments (Goode et al., 1999). Genetic analyses have revealed that these proteins regulate microtubule-based cellular functions, such as nuclear migration and axonal guidance (Heil-Chapdelaine et al., 1998; Goode et al., 1999). These studies taken together indicate that coronins play an important role in actin and microtubule-based processes. However, despite the presence of these proteins in a number of lower eukaryotes, including *Plasmodium falciparum* (Tardieux et al., 1998), *Trichomonas vaginalis* (Bricheux et al., 2000), *Babesia species* (Figueroa et al., 2004), *Acanthamoeba healyi* (Baldo et al., 2005) and *Leishmania donovani* (Nayak et al., 2005), little is known about their functions in these organisms.

Leishmania are an important group of flagellated kinetoplastid parasites that are transmitted to humans by the bite of sand fly. They cause a wide spectrum of human diseases, including Kala-azar (Desjeux, 2004). Unlike other eukaryotes, the cytoskeleton of these pathogens is marked by a dense microtubular corset that surrounds the entire cell body and defines their shape. However, the flagellar pocket and distal tip regions are devoid of the microtubular framework. The flagellar pocket is the only site that facilitates endocytosis, recycling of cell surface molecules and accumulation of several membrane-bound receptors (Bonhivers et al., 2008; Krishnamurthy et al., 2005; Hung et al., 2004). Although various cytoskeletal proteins become redistributed to the distal tip during cell division (Kratzerova et al., 2001; Gerald et al., 2007), no specialized functions have so far been attributed to this site.

Previously, we identified a novel homolog of coronin in *Leishmania* species (Nayak et al., 2005), which belongs to the phylogenetically oldest clade of coronin proteins, and has recently been renamed into CRN12 (Morgan and Fernandez, 2008). We also reported that *Leishmania* CRN12 associates with filamentous actin in *Leishmania* promastigotes (Nayak et al., 2005; Kapoor et al., 2008), and is retained in the flagellar pocket region and in a few cells at the distal tip (Nayak et al., 2005). We have now further explored the intracellular distribution of *Leishmania* CRN12 in both the resting and dividing *Leishmania* cells. In addition, we have generated *Leishmania* CRN12 deletion mutants, and studied the effects of CRN12 depletion on the growth and cell division of the mutant cells. The results reported here indicate that *Leishmania* CRN12 plays a crucial role in corset-microtubule remodeling during cytokinesis.

Results

Leishmania CRN12 accumulates at the posterior ends in dividing cells and co-localizes with tubulin and kinesin K39

Our earlier studies have shown that CRN12 in detergent-treated *Leishmania* promastigotes is retained in the flagellar pocket region and also in a few cells at the posterior pole (Nayak et al., 2005). To explore this further, we analyzed the intracellular CRN12 distribution in both the resting and dividing *Leishmania* promastigotes. The analyses revealed that CRN12 was invariably concentrated at the posterior ends throughout cytokinesis until the final stage of separation of the daughter cells (Fig. 1B). However, in resting cells, CRN12 accumulation at the posterior end was not a regular feature as only about 27% (26.9±5.6%, n=1780) of these cells showed posterior accumulation (Fig. 1A). Interestingly, in the CRN12 accumulation zones, actin was only faintly stained. As posterior ends are rich in dynamic microtubules, we analyzed whether *Leishmania* CRN12 was present together with the dynamic microtubules at these sites. The dynamic microtubules can be stained with tyrosinated α-tubulin antibodies (YL1/2) that specifically recognize the newly synthesized microtubule ends (positive ends) (Tu et al., 2005). Immunofluorescence analyses showed specific colocalization of CRN12 with dynamic microtubules at the posterior ends of dividing cells (Fig. 2A). Furthermore, a microtubule-based motor kinesin K39 has been shown to have an intracellular distribution (Gerald et al., 2007) similar to that observed here for CRN12 in dividing *Leishmania* promastigotes. It was therefore logical to examine whether CRN12 is associated with kinesin K39 during cytokinesis. Interestingly, this protein also colocalized with CRN12 at both the anterior and posterior ends of the dividing cells, whereas in the resting cells their colocalization was limited only to the anterior end (flagellar pocket region) (Fig. 2B). These results demonstrated that *Leishmania* CRN12 redistributed in a cell cycle-dependent manner and associated with growing ends of microtubules and microtubule-based motor kinesin K39 directly or indirectly. Earlier studies with *S. cerevisiae* CRN11 have shown that this protein binds microtubules through a microtubule-binding motif present in its variable region (Goode et al., 2000). Although *Leishmania* CRN12 contained a variable region in between WD repeats and C-terminal coiled coil region, the microtubule binding motif was not distinguishable in its primary structure. It is therefore likely that, unlike *S. cerevisiae* CRN11, *Leishmania* CRN12 was associated with microtubules indirectly, perhaps through kinesin K39 (Gerald et al., 2007). To examine the validity of this proposal, we analyzed the presence of CRN12 in microtubule cytoskeleton preparation as well as in the materials obtained after immunoprecipitation of the promastigote lysate supernatant fraction with anti-tubulin antibodies or anti-rK39 antibodies (Fig. 2C,D). CRN12 was present in both the microtubule cytoskeleton and the cytoplasm. However, only anti-rK39 antibodies could precipitate CRN12 from the cytosolic fraction, suggesting that CRN12 was associated with microtubules through kinesin K39.

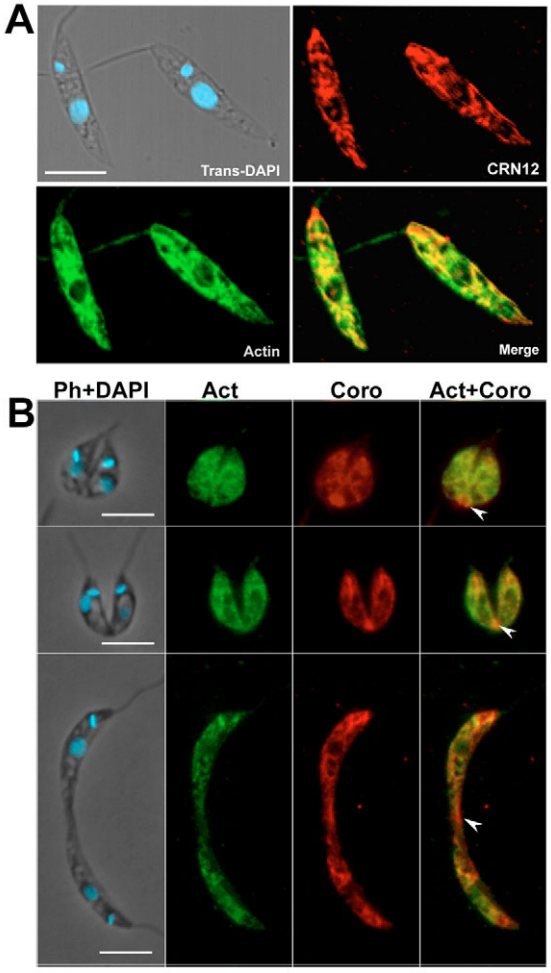

Fig. 1. Intracellular distribution of CRN12 and actin in resting and dividing *Leishmania* promastigotes. (A) Immunofluorescence images showing colocalization of CRN12 and actin at most of the places and a predominant localization of CRN12 in the flagellar pocket region in the resting cells. (B) Immunofluorescence images showing localization of both CRN12 and actin in the dividing cells. All these cells show a characteristic pattern of CRN12 accumulation at both the flagellar end and posterior pole. Arrowheads indicate posterior pole accumulation of CRN12 in the dividing cells. Ph, phase; Trans, transmission; *Coro*, CRN12; Act, actin. Scale bars: 5 μm.

Apart from the posterior end, CRN12 was also colocalized with kinesin K39 in the anterior region just above the kinetoplast DNA, which was suspected to be the basal body region. Recently, duplication of the basal body has been shown to be crucial for the survival of *Leishmania* (Selvapandiyan et al., 2004). We therefore analyzed whether CRN12 was associated with the basal body complex that could be marked by immunostaining centrin (Selvapandiyan et al., 2004). Interestingly, CRN12 also colocalized with centrin in the basal body region, but, unlike centrin, its distribution deviated from the flagellar fibrils (Fig. 3). These results suggested that *Leishmania* CRN12 might be an integral component of the basal body, as well as of some other undefined structural component in the flagellar pocket region. As actin has been shown to be present in this region (Sahasrabuddhe et al., 2004), we carefully examined colocalization of CRN12 with actin in the detergent-treated cells. Our analysis revealed only a limited co-localization of actin with CRN12 in the region outside the basal body. This suggested an actin independent role of CRN12 with another tubulin based structure – 'the basal body complex'.

Targeted CRN12 gene replacement in *L. donovani*

CRN12 is a single copy gene in *Leishmania* (Berriman et al., 2005) that we re-confirmed in our *L. donovani* strain (see supplementary material Fig. S1). The two alleles of *L. donovani* CRN12 gene were sequentially replaced with two different antibiotic resistance markers, neomycin phosphotransferase (Neo) and hygromycin

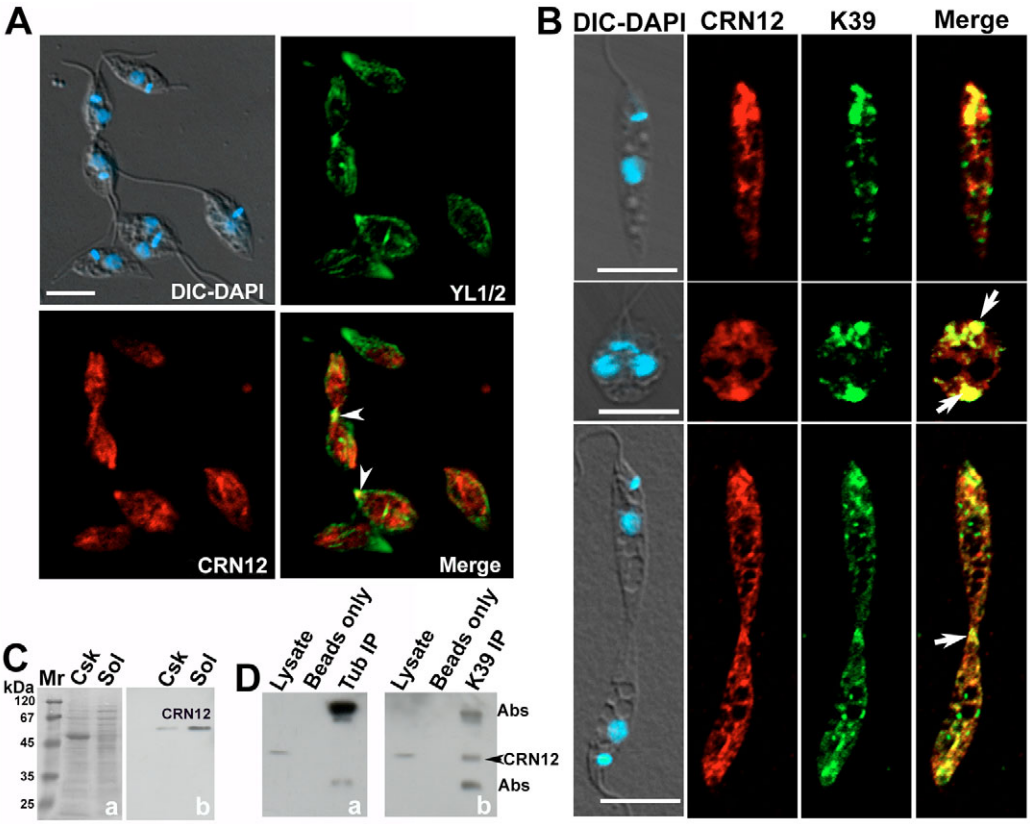

Fig. 2. Interactions of CRN12 with microtubules and kinesin K39. (A) Immunofluorescence images showing colocalization of CRN12 with positive ends of microtubules stained with YL1/2 antibodies. Unlike resting cells, the dividing cells show clear colocalization with microtubules (arrowheads) at their posterior poles. Scale bar: 5 µm. (B) Immunofluorescence images of resting and dividing cells labeled for CRN12 and kinesin K39. Arrows show colocalization of both the proteins in the flagellar pocket and posterior pole regions in the dividing cells. Notably, no such co-localization at the posterior pole in the resting cells is visible. Scale bars: 5 µm. (C) Western blot analysis showing presence of CRN12 in the microtubule cytoskeleton preparation. (a) Coomassie blue stained blot; (b) western blot of (a) probed with anti-CRN12 antibodies. Csk, detergent insoluble microtubule cytoskeleton; Sol, soluble cytosolic fraction; M_r, molecular weight markers. (D) Western blot analyses of tubulin and rK39 immunoprecipitates (IP) for the co-presence of CRN12. (a) Immunoprecipitate, obtained using anti-tubulin antibodies, probed with anti-CRN12 antibodies; (b) immunoprecipitate, obtained using anti-rK39 antibodies, probed with anti-CRN12 antibodies.

phosphotransferase (Hyg) genes, by homologous recombination that conferred resistance to antibiotic G-418 and hygromycin B, respectively. The targeted constructs contained 650 bp upstream and 950 bp downstream regions of the CRN12 gene at the 5′ and 3′ ends of the antibiotic resistance marker (Hyg or Neo) genes, respectively (Fig. 4A).

In the first round, the deletion cassette containing Neo gene was transfected in *L. donovani* by electroporation (Tammana et al., 2008). The heterozygous clones, selected against 50 µg/ml G418 on DMEM-agar plates, appeared after 20-25 days of transfection. Genotypic analyses of these clones by Southern blotting confirmed the integration of the deletion cassette at the CRN12 locus (see supplementary material Fig. S2A). These results were reconfirmed by PCR using one primer from the Neo gene (P1 in Fig. 4A) and another from the downstream region of the 3′flank used in the deletion construct (P2 in Fig. 4A), which specifically amplified the desired 2.1 kb DNA (see supplementary material Fig. S2B). Reduction in the protein expression levels was analyzed by western blotting (see supplementary material Fig. S2C), which showed about 40-50% reduction in CRN12 levels in the heterozygous mutants ($Coro^{+/-}$) compared with the wild-type ($Coro^{+/+}$) cells (see supplementary material Fig. S2D).

Double allele replacement of CRN12 gene yields only aneuploid cells

A heterozygous clone, with one allele of CRN12 gene replaced by the Neo gene, was used for the second round of transfection with the deletion cassette containing the Hyg gene. The colonies of doubly selected clones ($Coro^{-/-}$) appeared on DMEM-agar plates containing 50 µg/ml G418 and 50 µg/ml hygromycin. Although a few colonies emerged within 20-25 days, several colonies kept emerging even up to 60 days of transfection. The clones selected at variable time periods, showed integration of both the Hyg and Neo cassettes at the CRN12 locus (see supplementary material Fig. S2E-H). However, despite the replacement of the two alleles of the CRN12 gene with Neo and Hyg genes, these clones showed the presence of CRN12 gene (see supplementary material Fig. S2E,F) and varied expression levels of CRN12 (Fig. 4B). Even after several attempts, we failed to isolate $Coro^{-/-}$ null mutants, which suggested changes in the ploidy (Martínez-Calvillo et al., 2005; Cruz et al., 1993) of the selected cells. However, the DNA content analyses of $Coro^{+/+}$, $Coro^{+/-}$ and $Coro^{-/-}$ clones by flow cytometry revealed that all the three cell types have similar DNA contents (Fig. 4C), suggesting that the selected clones could be aneuploids that possessed an extra copy or copies of the chromosome containing

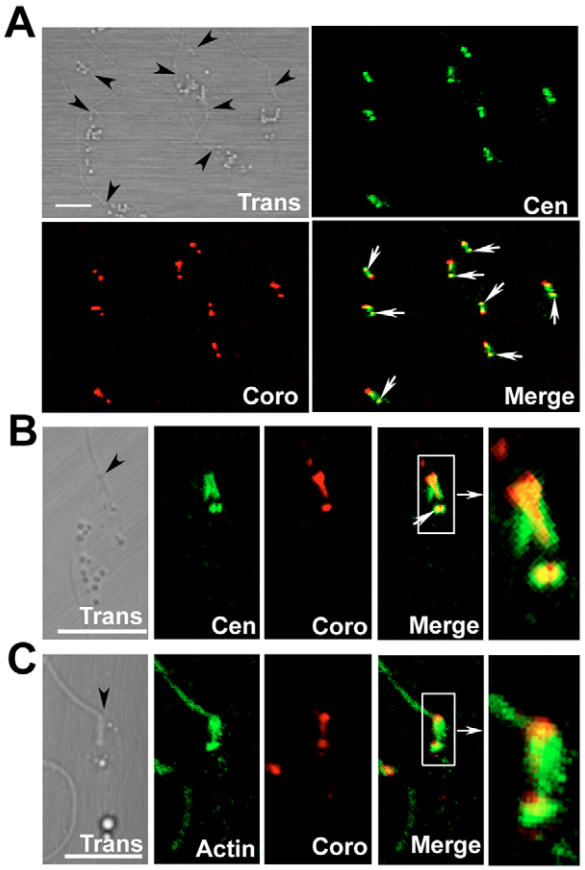

Fig. 3. Localization of CRN12 in the basal body region marked by centrin in the NP-40-treated (0.5%, v/v) *Coro*$^{+/+}$ cells. (A) Immunofluorescence images showing colocalization of CRN12 and centrin in the basal body region. Arrows indicate the colocalization. (B) Immunofluorescence image of a magnified cell cytoskeleton showing presence of CRN12 in the basal body region and its deviation from the flagellar fibrils stained with centrin antibodies. (C) Immunofluorescence image of the cytoskeleton stained for CRN12 and actin to show their different locations in the flagellar pocket region. Arrowheads indicate flagellar pocket regions of the promastigote cytoskeleton in the transmission images. Boxed regions are magnified further for more clarity. Arrows indicate the colocalization. Trans, transmission; Cen, centrin; *Coro*, CRN12. Scale bars: 5 µm.

the CRN12 gene. We also observed a slight increase in the G2/M cell population, which could be due to the observed presence of bipolar cells in the *Coro*$^{+/-}$ and *Coro*$^{-/-}$ cultures.

Coro$^{-/-}$ null cells fail to survive in culture

Having failed to obtain homozygous *Coro*$^{-/-}$ null mutants through standard procedures, we followed doubly transfected cells in liquid cultures. In the presence of 50 µg/ml G-418 and 50 µg/ml hygromycin, *Coro*$^{+/-}$ cells (G418 resistant) were selected within 8-10 days under our experimental conditions. Therefore, the doubly transfected cells were analyzed after 10 days of their selection. As very few cells survived the selection process in the presence of G-418 and hygromycin, genotypic analysis of these cells by Southern blotting was not possible. Therefore, a small population of the selectants was harvested at different time intervals and then subjected to western blot analysis (see supplementary material Fig. S2I). At the initial stages, CRN12 concentration was markedly reduced to negligible levels but slowly increased to normal levels with subsequent reduction in the occurrence of bipolar cells (see supplementary material Fig. S3) with time. The presence of CRN12 gene was analyzed by PCR, which showed poor amplification on the 18th day when compared with the 24th day of transfection (see supplementary material Fig. S2J) and also the integration of the Neo and Hyg deletion constructs at the CRN12 gene locus (see supplementary material Fig. S2K). Microscopic observation at various time intervals revealed that majority of the cells during those 18-21 days had sluggishly beating flagella and were largely immotile. Furthermore, most of these cells had normal cell morphology, but a few cells also appeared rounded in shape and, over time, were superseded by the motile cells (data not shown). These results suggested that *Coro*$^{-/-}$ null cells failed to survive long in culture.

Coro$^{+/-}$ cells show bipolar-cylindrical morphology and reduced growth

In a general microscopic view of the *Coro*$^{+/-}$ cultures, many cells appeared in a unique bipolar-cylindrical morphology (Fig. 5A) with one actively wriggling flagella at each end. Such cells represent ~28% (n=574-2932 cells) of the cells in the log phase *Coro*$^{+/-}$ cultures, when compared with only <1% in the *Coro*$^{+/+}$ cells. These bipolar *Coro*$^{+/-}$ cells possessed two kinetoplasts and two nuclei in proportion to the two flagella and, therefore, appeared to be the fusion of two cells at their posterior ends (Fig. 5A). The presence of a set of kinetoplast and nucleus for each flagellar end suggested that these cells had successfully completed kinetoplast and nuclear division. It was also noticeable that a few of these bipolar cells underwent another cycle of division before undergoing separation of daughter cells, producing tetra-polar cells (4.2±1.12% of total cells). However, the ratio of nuclei, kinetoplasts and flagella remained the same (see supplementary material Fig. S4). Interestingly, these abnormalities disappeared in stationary phase cultures, suggesting the phenomenon to be highly growth-phase specific. These findings were further corroborated with the growth of *Coro*$^{+/-}$ cells where reduction in the growth rate was peculiar to the log phase (Fig. 5B), which could be due to delayed separation of the daughter cells.

Episomal expression of CRN12 rescues bipolar defect in *Coro*$^{+/-}$ cells

To unequivocally establish that the bipolar defect was related to the reduction in CRN12 levels, *Coro*$^{+/-}$ cells were transfected with episomal expression vector p6.5MCS containing CRN12 gene (p6.5MCS-CRN12), using p6.5MCS vector alone transfected cells as the control. Cells were selected against 20 µg/ ml tunicamycin and 50 µg/ ml neomycin. The increased expression level of CRN12 in the complemented cells was confirmed by western blotting (Fig. 6). These cells showed higher growth and significant decrease in the number of bipolar cells, compared with the control cells (Fig. 6), confirming that the increased formation of bipolar cells in *Coro*$^{+/-}$ cultures was primarily due to the reduction in CRN12 levels.

Rhizoxin inhibits formation of bipolar-cylindrical cells

As cell shape in trypanosomatids is regulated by the microtubule cytoskeleton (Gull, 1999), the observed bipolar cell morphology of *Coro*$^{+/-}$ cells could be attributed to uncontrolled growth of corset microtubules. To examine this fact, we analyzed the shape of the *Coro*$^{+/-}$ cells grown in the presence of the microtubule inhibitor, rhizoxin, which has earlier been used to inhibit microtubule

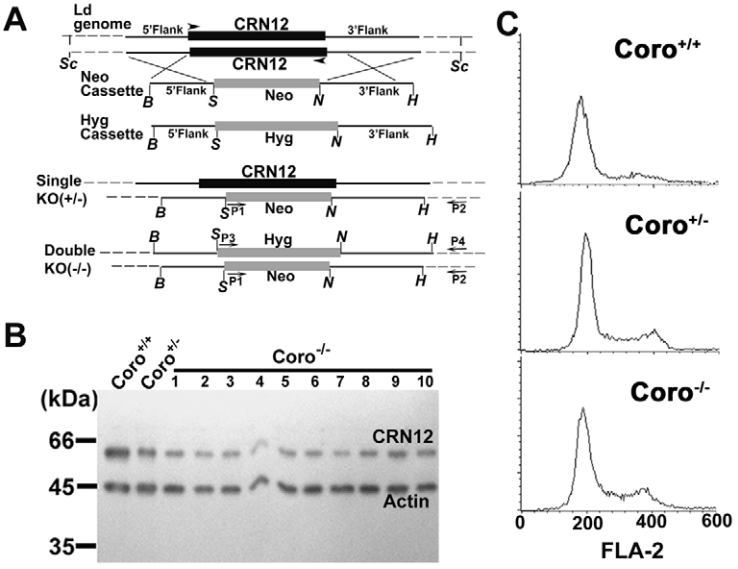

Fig. 4. (A) DNA constructs of *Leishmania* CRN12 gene deletion cassettes. Schematic representation of CRN12 locus in *L. donovani* before and after integration of Neo and/or Hyg cassettes, and incorporation of restriction sites *Bam*HI (*B*), *Sal*I (*S*), *Not*I (*N*) and *Hin*dIII (*H*). *Sc* indicates *Sac*I sites in the genome adjacent to the CRN12 locus. Sites for primers P1-P4, used for the confirmation of Neo and Hyg cassette integration and amplification of CRN12 (arrowheads) by PCR, are also shown. (B) Western blot analysis of CRN12 mutants. Lanes 1-10, 10 $Coro^{-/-}$ clones showing varied levels of CRN12 expression. (C) Flow-cytometry analysis of $Coro^{+/+}$, $Coro^{+/-}$ and $Coro^{-/-}$ cells showing similar DNA contents with 2.62%, 10.86% and 6.84% G2/M populations, respectively, as determined by ModFit program (version 3.0).

assembly in trypanosomatids (Tu et al., 2005). Addition of rhizoxin to the $Coro^{+/-}$ cultures inhibited emergence of the bipolar-cylindrical cells (see supplementary material Fig. S5). In addition, a set of $Coro^{+/-}$ cultures that were given rhizoxin treatment after emergence of the bipolar-cylindrical cells (day 6) showed a significant drop in the number of such cells within 8 hours and their complete elimination after 24 hours of the treatment. These results suggested that the formation of bipolar-cylindrical cells was primarily due to the uncontrolled elongation of corset microtubules, which was consistent with the earlier study which showed that knockdown of yeast CRN11 results in long cytoplasmic microtubules (Goode et al., 1999).

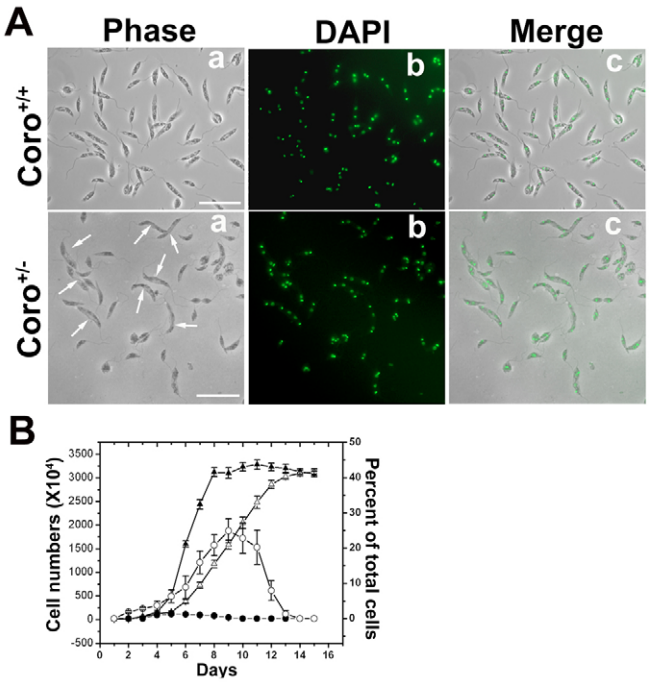

Fig. 5. Effect of CRN12 depletion in $Coro^{+/-}$ *Leishmania* promastigotes on their morphology and growth. (A) Microscopic images of $Coro^{+/+}$ and $Coro^{+/-}$ cells showing the presence of a significant number of bipolar cells in $Coro^{+/-}$ cell population. (a) Phase contrast; (b) DAPI-stained nuclei and kinetoplasts; (c) merge of 'a' and 'b'. Arrows indicate the bipolar cells; scale bars: 20 µm. (B) Growth curve of $Coro^{+/+}$ (black triangles) and $Coro^{+/-}$ cells (white triangles) showing reduction of growth rate in the log phase and appearance of bipolar cells in heterozygous mutants (black circles, $Coro^{+/+}$ cells; white circles, $Coro^{+/-}$ cells). Values shown are means of three independent experiments ± s.d.

$Coro^{+/-}$ cells show defects in corset microtubule reorganization during cytokinesis

The generation of bipolar-cylindrical morphology in $Coro^{+/-}$ cells prompted us to analyze the positive end dynamics of their corset microtubules. Immunofluorescence images of the bipolar cells stained with tyrosinated α-tubulin antibodies showed clustering of the microtubule positive ends at different positions, including the middle region and flagellar ends (Fig. 7A, part b). A number of cells also showed uniform punctuated distribution of these ends throughout the cell. As these positive ends distinctly marked the posterior end in control cells (Fig. 7A, part a), formation of the bipolar cells could be due to intrusion of the persistently growing corset microtubules into the other daughter cell corset from the opposite direction. A representation of these images in a plausible series of events indicated defective regulation of the corset microtubules during cytokinesis in $Coro^{+/-}$ cells. To further confirm these findings, we analyzed the negatively stained cytoskeletons of the bipolar cells by transmission electron microscopy (Fig. 7B, part a). Substantiating the results of the immunofluorescence analysis, the corset microtubules in the bipolar cells were continuous from one flagellar end to the other and their numbers increased significantly (100-104 microtubules, n=4 cells) when compared with the normal cells (48-74 microtubules, n=9 cells). As the bipolar cells appeared to have been formed from microtubules from the two daughter cells, originating from opposite directions, it could be assumed that arrangement of the microtubules in the corset was anti-parallel. Despite the anti-parallel arrangement, the interlinking pattern of these microtubules was similar to that of the wild-type cells (Fig. 7B, parts b,c). These results suggested that during cytokinesis of the $Coro^{+/-}$ cells, microtubules grew in an unrestricted manner and eventually invaded the other daughter cell corset, thereby generating the bipolar-cell morphology.

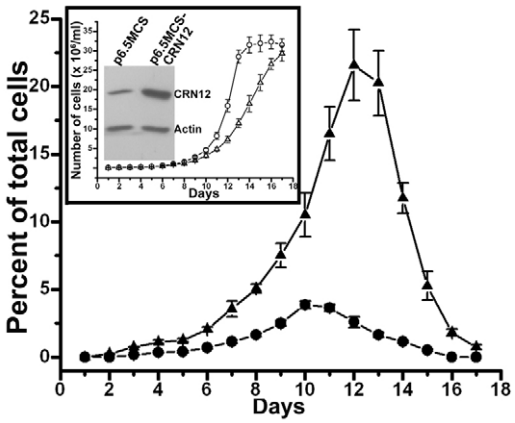

Fig. 6. Analyses of bipolar cell formation and cell growth after episomal complementation of CRN12 in *Coro*$^{+/-}$ cells. Black triangles and black circles indicate appearance of bipolar cells in p6.5MCS vector and p6.5MCS-CRN12 transfectants, respectively. Inset shows growth curve of p6.5MCS and p6.5MCS-CRN12 transfectants (white triangles and white circles, respectively), and western blot of both the transfectants showing increased CRN12 expression levels (2.16±0.26-fold, n=3) in p6.5MCS-CRN12 transfectants when compared with p6.5MCS transfectants. Densitometric values of CRN12 were normalized with actin (internal loading control).

Intracellular distribution of CRN12 in *Coro*$^{+/-}$ cells having bipolar morphology

Given that only 25-30% cells adopt bipolar morphology, many of the *Coro*$^{+/-}$ cells would obviously divide through the normal mode. In the normally dividing *Coro*$^{+/-}$ cells, CRN12 was found to concentrate and colocalize with tubulin at their posterior ends (Fig. 8A). However, in the bipolar cells presence of CRN12 in the middle region was scarce (Fig. 8B). Similar to CRN12, kinesin K39 was also absent from this region (Fig. 8C). As the level of CRN12 expression was apparently low in *Coro*$^{+/-}$ cells, the residual CRN12 was present mainly towards the flagellar end, where, to some extent, it colocalized with centrin in the basal body (Fig. 8D).

As no dead bipolar cell could be seen in the late log and stationary phase cultures, where the number of such cells is considerably decreased, it is intriguing as to how these cells managed separation of their daughter cells. To analyze this problem, we carefully examined the late log phase *Coro*$^{+/-}$ cells (when the number of bipolar cells decline) after their labeling with YL1/2 antibodies. Interestingly, the bipolar cells appeared to have clearly demarcated two daughter cell corsets that tend to separate towards the opposite poles by some traction force exerted perhaps by the flagellar movement (see supplementary material Fig. S6).

Discussion

This study presents a functional characterization of a coronin homolog (CRN12) in the human pathogen *L. donovani*. We show here that targeted replacement of one allele of *Leishmania* CRN12 gene by the marker gene results in generation of bipolar cells, specifically during the log phase, owing to unregulated growth of corset microtubules. This effect is reversed by episomal gene complementation and the cells where both the alleles of the CRN12 gene are replaced by the marker genes do not survive in culture. We further show that CRN12 interacts with dynamic microtubules through a microtubule-based motor, kinesin K39, at the posterior pole in a cell cycle-dependent manner and also colocalizes with the basal body protein centrin in the basal body region. These results demonstrate that CRN12 plays a crucial role in microtubule remodeling during *Leishmania* cytokinesis.

Cytokinesis in trypanosomatids is initiated from the anterior end and terminates at the posterior end followed by daughter cell separation (Ploubidou et al., 1999). During this process, the newly synthesized corset microtubules grow along with the furrow ingression and terminate towards the posterior pole (Hammarton et al., 2007) [towards which their positive ends are aligned (Robinson et al., 1995)]. Spindle shapes of the cells are attained by restricting the corset microtubules at various places, thereby reducing their numbers towards the posterior pole (Rindisbacher et al., 1993). However, emergence of the unique bipolar-cylindrical, fused daughter cell morphology after CRN12 depletion in heterozygous mutants suggests that the corset microtubules grow in an unrestricted manner and apparently invade into the other daughter cell corset during cytokinesis. Earlier studies have shown that polymerization of the microtubules at the posterior end is well coordinated with the cell cycle in trypanosomatids (Hendriks et al., 2001; Matthews 2005; Tu and Wang, 2005; Tu et al., 2005). These ends have been suggested to be important foci for cytoskeletal organization in trypanosomatid parasites during their growth, development and differentiation (Gerald et al., 2007). Putative genes for the positive-end microtubule capping proteins EB1 and Bim1P have been detected in the genomes of *T. brucei* and *T. cruzi*, but no orthologs of these genes are detectable in the *L. major* genome (Berrimen et al., 2005). It would, therefore, seem that some unconventional proteins might have taken over these functions in *Leishmania*. Several proteins have been shown to localize at the posterior pole, which appear to orchestrate microtubule capping as well as plugging by some as yet unidentified mechanism (Rinidispacher et al., 1993; Baqui et al., 2000; Kratzerova et al., 2001; Libusova et al., 2004; Gerald et al., 2007). Some of these proteins specifically concentrate at this pole only during cytokinesis (Kratzerova et al., 2001; Gerald et al., 2007), suggesting their dynamic role in organization of the cap and/or plug assemblies. As CRN12 redistributes to the posterior pole during cell division and interacts with dynamic microtubules through a microtubule-based motor, kinesin K39, we suggest that this protein could play an important role in the posterior pole morphology.

Physiological activities at the posterior pole during cell division can be divided into three major events: (1) microtubule capping to limit their growth; (2) plugging the positive ends together; and (3) scission of the two daughter cells through fusion of the posterior pole membrane. Our immunofluorescence analysis locating positive ends of microtubules in the bipolar-cylindrical cells reveals that the microtubules in the furrow ingression proceed towards the posterior pole and eventually invade the other daughter cell corset. However, it remains still unclear how the growth of these unregulated microtubules is stopped after approaching the flagellar end of the other daughter cell. Perhaps, physico-spatial limitations, together with residual microtubule-capping activities, constrain their growth.

Characteristic spiral arrangement of microtubules in the corset has been suggested to be regulated through various microtubule binding proteins that interlink corset microtubules (Bramblett et al., 1987). The fused bipolar cells showed no divergence from this pattern and, therefore, the role of CRN12 in spatial arrangement of corset microtubules can be ruled out. Furthermore, electron microscopic analysis of the bipolar cell cytoskeleton suggested that the microtubule cross-linking proteins equally recognize both the parallel (from the same cell) and anti-parallel (from both the

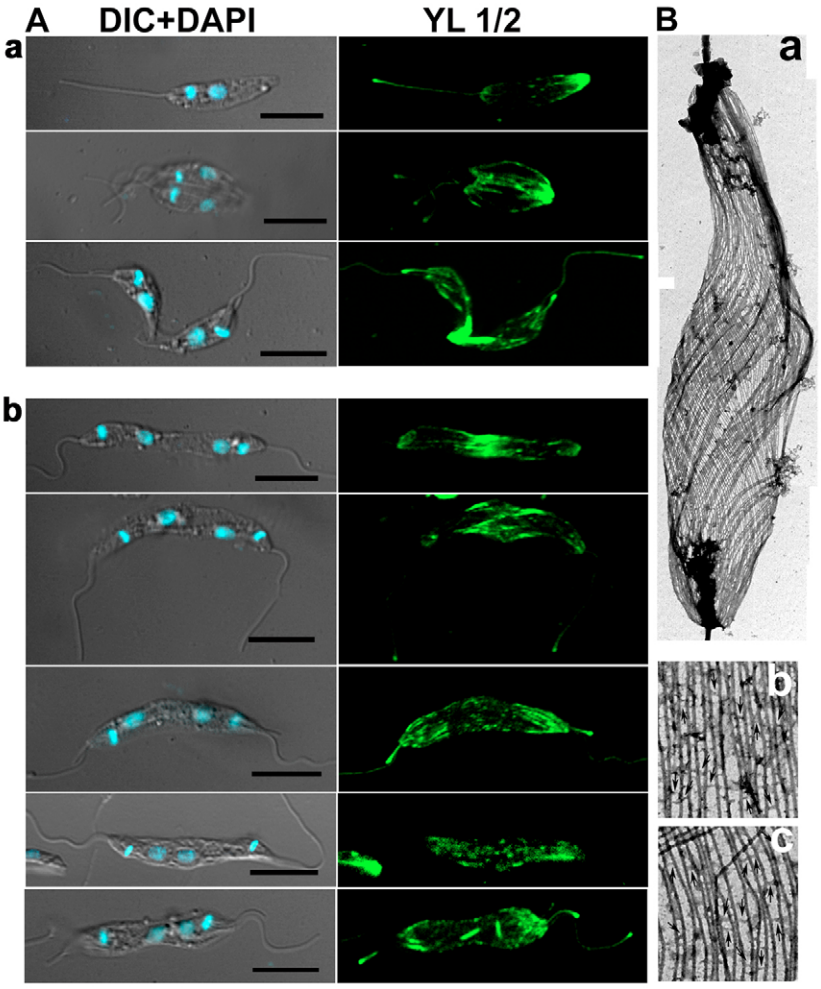

Fig. 7. Analysis of microtubule organization in the bipolar-cylindrical *Coro*$^{+/-}$ cells. (A) Labeling of microtubule positive ends by anti tyrosinated α-tubulin antibodies (rat monoclonal YL1/2). (a) Positive end labeling that specifically marks posterior pole of the resting and dividing *Coro*$^{+/+}$ cells; (b) positive end clustering at different locations in *Coro*$^{+/-}$ cells with bipolar morphology. Scale bars: 5 µm. (B) Negatively stained transmission electron microscopic image of Triton X-100 treated *Coro*$^{+/-}$ cell with bipolar morphology, showing its microtubular organization. (a) Manually reconstituted electron micrograph of eight images taken at high magnification clearly showing continuity of corset-microtubules from one flagellar end to the other. (b,c) Negatively stained corset-microtubules showing their interlinking patterns in the control cell and bipolar cell, respectively. Arrows indicate sites of interlinking.

daughter cells) arrangement of microtubules in the corset. In fact, it is this indiscriminate interlinking of parallel and anti-parallel microtubules that causes the length of the bipolar cells to be restricted to only twice the length of one *Leishmania* promastigote. Under conditions of seemingly unrestricted microtubule growth, bipolar cells would probably be over twice the length of one *Leishmania* promastigote.

Interaction of coronin with microtubules has been reported in *S. cerevisiae*, where CRN11 (a coronin homolog) is shown to link the actin network to the microtubules by its binding through a microtubule-binding motif present in its variable region (Heil-Chapdelaine et al., 1998; Goode et al., 1999; Goode et al., 2000). However, *Leishmania* CRN12 interacts with dynamic microtubules indirectly through kinesin K39. The gene database of *Leishmania* reveals a plethora of kinesins that are basically microtubule-based motors (Berrimen et al., 2005). As these organisms contain microtubules as their major cytoskeleton component, these kinesins are believed to perform diverse functions in the trypanosomatids cell physiology (Berrimen et al., 2005). Phylogenetic analysis has revealed that kinesin K39 clusters near the kinesin 3 family (Dagenbach and Endow, 2004; Miki et al., 2005; Wickstead and Gull, 2006) and one of the kinesin 3 family proteins binds myosin IIA, providing an interface between the actin and microtubule cytoskeletons (Kopp et al., 2006). It is, therefore, possible that association of actin-binding protein CRN12 with a microtubule-based motor, kinesin K39, might provide a link between the actin and microtubule cytoskeleton in *Leishmania*.

The *Coro*$^{-/-}$ null mutants retain their normal cell morphology, possibly because of their inability to enter into the cell division cycle. As CRN12 colocalizes with centrin, which plays an important role in basal body organization and duplication, we speculate that the observed failure of *Coro*$^{-/-}$ null mutants to survive in culture is due to the essential requirement of CRN12 in basal body duplication that has been shown to be crucial for the commencement of cell division in *Leishmania* (Selvapandiyan et al., 2004; Hammarton et al., 2007). Furthermore, survival of the *Coro*$^{-/-}$ null mutants by possible expansion of their chromosome number suggests that CRN12 is essential for survival and multiplication of *Leishmania* in culture. Although the mechanism by which the ploidy changes occur upon targeted gene disruption is not known, this phenomenon has been widely accepted as the confirmation of essentiality of the gene being targeted (Mottram et al., 1996; Cruz et al., 1993; Dumas et al., 1997; Tovar et al., 1998).

Finally, very little is known about the proteins that regulate the various steps during *Leishmania* cytokinesis (Hammarton et al., 2007). Results presented here, for the first time, show that an actin binding protein CRN12, in association with a microtubule-based motor kinesin K39, plays a crucial role in the remodeling of corset-microtubules during *Leishmania* cytokinesis, and suggest that this protein may also be required in basal body duplication.

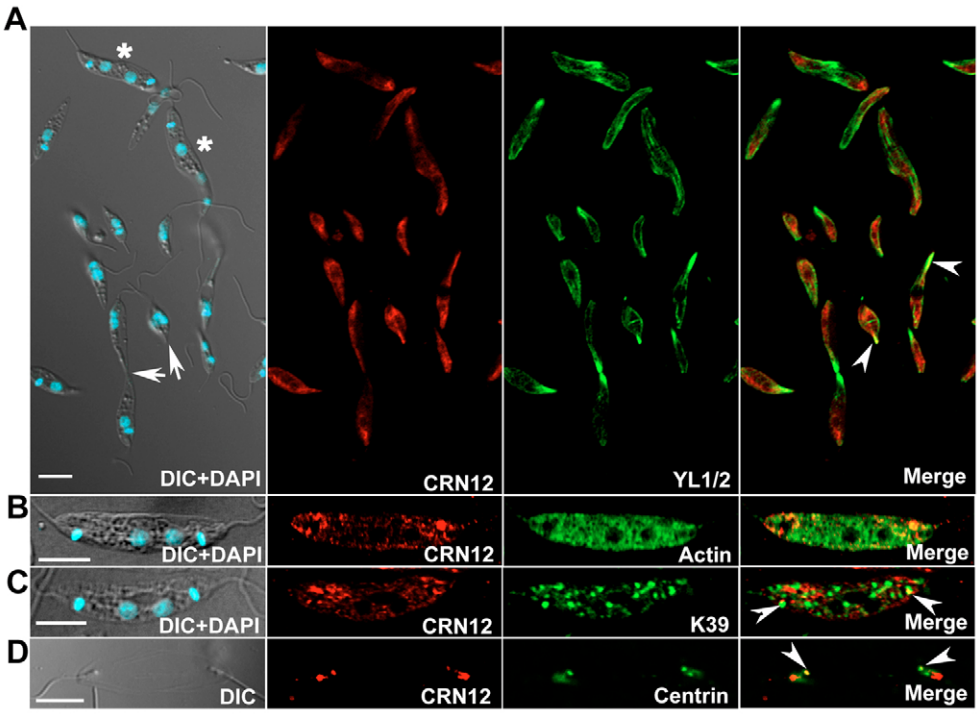

Fig. 8. Localization of CRN12, tubulin, kinesin K39, centrin and actin in the *Coro*$^{+/-}$ cells. (A) Immunofluorescence images of a field showing different stages of the *Coro*$^{+/-}$ cells and distribution of CRN12 and positive ends of microtubules. Asterisks indicate bipolar cells, arrows indicate normally dividing cells and arrowheads indicate colocalizations of CRN12 and positive ends of microtubules. (B) Immunofluorescence images showing distribution of CRN12 and actin in bipolar cells. (C) Immunofluorescence images showing distribution of CRN12 and kinesin K39. These two proteins colocalize at various places but do not concentrate in the middle region of the bipolar cell. Arrowheads indicate colocalization. (D) Immunofluorescence images of bipolar cell cytoskeleton showing conserved locations of CRN12 in the basal body region marked by centrin. Arrowheads indicate colocalization. For clear presentation of CRN12 colocalization, images have been collected at slightly increased gain settings and are not quantitative.

Materials and Methods

Cell culture

Leishmania donovani (Dd8) strain was obtained from National Institute of Immunology, New Delhi (India) and maintained in high glucose DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 40 μg/ml gentamycin at 25°C. The single and double allele CRN12 deletion mutants of *L. donovani* cells were grown in the above growth medium after inclusion of 50 μg/ml G-418 and/or 50 μg/ml hygromycin, respectively.

Antibodies

Anti *Leishmania* recombinant CRN12 antibodies in mice and rabbits and anti *Leishmania* recombinant actin antibodies in rabbits were raised and purified as described earlier (Nayak et al., 2005; Sahasrabuddhe et al., 2004). Antibodies against α,β-tubulins (monoclonals) and anti-tyrosinated α-tubulin antibodies were procured from ICN and Chemicon (USA), respectively. Anti-centrin antiserum and anti-rK39 antiserum were kind gifts from Hira Lal Nakhasi (Center for Biologics Evaluation and Research, FDA, Bethesda, MD) and Kwang-Poo Chang (Chicago Medical School, Rosalind Franklin University, North Chicago, IL), respectively.

Immunoprecipitation

Log-phase cultured promastigotes (10^8) were lysed in PME buffer (100 mM PIPES, 1 mM $MgSO_4$ and 0.1 mM EGTA; pH 6.9) containing 0.5% NP-40 on ice for 5 minutes and centrifuged at 14,000 × ***g*** for 30 minutes at 4°C. Soluble fraction was incubated with protein-A/G agarose beads and to the cleared supernatants were added anti-tubulin or anti-rK39 antibodies followed by treatment with protein-A/G agarose beads. For control purposes, protein-A/G agarose beads were separately incubated with the lysate. Proteins associated with the beads were released in SDS-sample buffer without any sulfhydryl reducing agent (2% sodium dodecylsulfate, 10% glycerol, 50 mM Tris-Cl, pH 6.8) at 95°C for 5 minutes and resolved on 10% SDS-polyacrylamide gel followed by western blotting using anti-CRN12 antibodies.

Flanking sequences and DNA constructs for targeted gene replacement

Upstream and downstream flanking sequences of *Leishmania* CRN12 gene were determined by using the earlier published procedures (Tammana et al., 2008). The flanking sequences were amplified by polymerase chain reaction (PCR) using primers UpF, DnF, UpR and DnR (see supplementary material Table S1) designed from the conserved upstream and downstream regions of CRN12 gene and *L. donovani* genomic DNA as the template. The amplicons were sequenced by Sanger's dideoxy chain termination method and sequences were submitted to EMBL (Accession numbers, FM242712 and FM242713).

The DNA constructs for targeted CRN12 gene replacement, consisted of three DNA fragments: (1) upstream flanking sequence (650 bp) of CRN12 gene; (2) genes for hygromycin phosphotransferase (Hyg) or neomycin phosphotransferase (Neo); and (3) downstream flanking sequence (950 bp) of CRN12 gene. The upstream flank of CRN12 gene containing *Bam*HI and *Sal*I restriction sites was cloned in InsT/Aclone vector, and a clone was selected with the *Sal*I site oriented towards the sp6 promoter, enabling us to use the *Hin*dIII site of the vector and named 'InsTA_5F'. Similarly, the downstream flank of CRN12 gene containing *Sal*I-*Not*I and *Hin*dIII restriction sites was cloned in InsT/Aclone vector and named 'InsTA_3F'. The downstream flank, released after the digestion with *Sal*I and *Hin*dIII, was ligated in 'InsTA_5F' pre-digested with the *Sal*I and *Hin*dIII, and the resulting construct was named 'InsTA_5-3F'. Finally, Neo and Hyg genes (from pXG-GFP2+/ and pCDNA3.1 vectors, respectively) containing *Sal*I and *Not*I restriction sites, were inserted in between the upstream and downstream flanks in 'InsTA_5-3F' at *Sal*I and *Not*I sites. The plasmid constructs thus generated were named as 'InsTA-Neo-KO' and 'InsTA-Hyg-KO', and were used further for the targeted replacement of CRN12 gene in *L. donovani* cells. For episomal complementation, the CRN12 gene was cloned in pMCS 6.5 vector (a kind gift from Kwang-Poo Chang) at *Nhe*I and *Hin*dIII restriction sites and named 'p6.5 MCS-CRN12'.

Genetic manipulations

Mid-log phase *L. donovani* promastigotes were transfected with 'InsTA-Neo-KO' and/ or 'InsTA-Hyg-KO' constructs as described earlier (Tammana et al., 2008). CRN12 single and double allele deletion mutants were selected against G-418 and/or hygromycin (Gibco-BRL) (50 μg/ml). Episomally complemented single CRN12 allele deletion mutants were selected against and maintained at 50 μg/ml neomycin and 20 μg/ml tunicamycin concentrations. For Southern hybridizations, genomic DNA (10 μg) from *Coro*$^{+/+}$, *Coro*$^{+/-}$ and *Coro*$^{-/-}$ cells was digested with *Sac*I enzyme, resolved on 0.8% agarose gel and transferred onto nylon membrane (Hybond N+, Amersham Pharmacia). Hybridization was carried out by using DIG-labeled probe prepared against CRN12, Neo and Hyg genes. Signals were detected using chemiluminescence substrate on an X-ray film.

Flowcytometry

Leishmania promastigotes were harvested by centrifugation at 3000 × ***g*** for 1 minute, resuspended in 100 μl PBS and injected into 1 ml pre-chilled (–20°C) methanol. After incubating for 3 minutes at –20°C, cells were centrifuged and resuspended in 1 ml of PBS containing 200 μg DNase free RNaseA (MBI Fermentas, Glen Burnie, MD) and incubated at 37°C for 30 minutes. Cells were collected by centrifugation and resuspended in PBS containing 10 μg/ml propidium iodide (PI) (Sigma) and DNA contents were analyzed on FACS Calibur (BD Biosciences) using ModFit program (version 3.0).

Immunofluorescence microscopy

Immunofluorescence microscopic analyses were carried out using earlier reported procedures (Nayak et al., 2005). To distinctly mark the presence of CRN12 in the basal body region, cells were treated with 0.5% NP-40, prior to their labeling with CRN12 and Centrin antibodies.

Electron microscopy

Whole cell cytoskeletons of *Leishmania* promastigotes were prepared by adhering the cells on the carbon-coated grids and washing with several drops of 1% Triton X-100 in Tris-buffered saline followed by staining with 1% (w/v) aqueous solution of uranyl acetate. The grids were analyzed under FEI Tecnai-12 Twin Transmission Electron Microscope equipped with a SIS Mega View II CCD camera at 80 kV (FEI, Hillsboro, OR).

Rhizoxin treatment

Coro$^{+/-}$ cells were seeded at 10^5 cells/ml concentration and rhizoxin (5 nM) was added either from the starting or on day 6 of cell seeding (when a significant number of bipolar cells start appearing) and number of bipolar cells were counted under optical microscope at 400× visual magnification.

This work was partly supported by the research grant awarded to C.M.G. by the Department of Biotechnology, Government of India, New Delhi (India), under the Distinguished Biotechnologist Award Scheme. Funding from Council of Scientific and Industrial Research (grant NWP0038) is also acknowledged. We thank Stephen M. Beverley for pXG-GFP2+/ vector, Kwang-Poo Chang for p6.5 MCS vector and anti-rK39 antibodies, and Hira Lal Nakahasi for anti-centrin antibodies. We are grateful to V. K. Bajpai and K. Mitra for electron microscopy. We thank A. L. Vishwakarma for FACS analysis. Finally, we are grateful to Saman Habib for critically going through the manuscript and useful suggestions. This is Communication No. 7641 From CDRI, Lucknow (India).

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