# *Leishmania* actin binds and nicks kDNA as well as inhibits decatenation activity of type II topoisomerase

Prabodh Kapoor<sup>1</sup>, Ashutosh Kumar<sup>1</sup>, Rangeetha Naik<sup>2</sup>, Munia Ganguli<sup>2</sup>, Mohammad I. Siddiqi<sup>1</sup>, Amogh A. Sahasrabuddhe<sup>1</sup> and Chhitar M. Gupta<sup>1,\*</sup>

<sup>1</sup>Division of Molecular and Structural Biology, Central Drug Research Institute, Chattar Manzil Palace, Lucknow-226001 and <sup>2</sup>Institute of Genomics and Integrative Biology, Delhi-110007, CSIR, India

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### ABSTRACT

Leishmania actin (LdACT) is an unconventional form of eukaryotic actin in that it markedly differs from other actins in terms of its filament forming as well as toxin and DNase-1-binding properties. Besides being present in the cytoplasm, cortical regions, flagellum and nucleus, it is also present in the kinetoplast where it appears to associate with the kinetoplast DNA (kDNA). However, nothing is known about its role in this organelle. Here, we show that LdACT is indeed associated with the kDNA disc in Leishmania kinetoplast, and under in vitro conditions, it specifically binds DNA primarily through electrostatic interactions involving its unique DNase-1-binding region and the DNA major groove. We further reveal that this protein exhibits DNA-nicking activity which requires its polymeric state as well as ATP hydrolysis and through this activity it converts catenated kDNA minicircles into open form. In addition, we show that LdACT specifically binds bacterial type II topoisomerase and inhibits its decatenation activity. Together, these results strongly indicate that LdACT could play a critical role in kDNA remodeling.

#### INTRODUCTION

Actin is a major cytoskeleton protein in eukaryotic cells, which primarily exists in two forms, viz: globular or monomeric form (G-actin) and filamentous or polymeric form (F-actin), and apart from its presence in the cytoplasm, it is also abundantly present in the nucleus (1). Whereas cytoplasmic actin is involved in a variety of cellular activities such as cell shape regulation, cell motility, endocytosis, exocytosis, intracellular trafficking and cytokinesis, nuclear actin is involved in transcription, nuclear export, intranuclear transport and chromatin remodeling (1,2). This protein in chromatin-remodeling complexes acts as a scaffold to incorporate other proteins which bind to DNA and exhibit ATPase, acetylase or helicase activity to remodel DNA in many processes such as transcription and DNA damage repair (3).

Besides nucleus, mitochondrion also contains its own autonomously replicating DNA, which encodes various mitochondrial proteins including those involved in the respiratory cycle. Normally, eukaryotic cells contain multiple copies of mitochondria, but the organisms that fall under the order Kinetoplastida such as Crithidia, Trypanosoma and Leishmania have only a single mitochondrion that contains an unusual genome known as kinetoplast DNA (kDNA). kDNA is a giant network of thousands of catenated circular DNAs that are organized in two types of circles, maxicircles and minicircles. Each kDNA is comprised of few dozens of maxicircles and several thousands of minicircles covalently interlocked in a compact kDNA disc (4). There is a general belief that the organization of kDNAs in all kinetoplastids is essentially the same, with only minor variations (5). Both the minicircles and maxicircles are relaxed, rather than supercoiled and are decatenated during the replication process that, unlike other eukaryotic cells where mitochondrial DNA replication occurs throughout the cell cycle, coincides with the S-phase of the cell cycle. This feature of mitochondrial DNA is unique to only this group of the eukaryotic organisms (6,7).

Leishmania belongs to the trypanosomatidae family and causes several human diseases including life threatening visceral leishmaniasis (8). These organisms mainly exist in two forms, viz: promastigotes and amastigotes. Whereas the flagellated promastigotes normally divide in the alimentary tract of the sand fly vector, the aflagellated amastigotes primarily exist and multiply within the mammalian macrophages (9). Although microtubules rather than microfilaments constitute the major cytoskeleton

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<sup>\*</sup>To whom correspondence should be addressed. Tel: 091 522 2610282; Fax: 091 522 2623405; Email: drcmg@sify.com

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network (10), actin and several actin-binding proteins are also abundantly present in *Leishmania* cells (11), some of which have already been shown to be required during the flagellar biogenesis and microtubule remodeling (12–14). Our recent studies have shown that *Leishmania* actin (LdACT) is a highly unconventional form of actin as unlike other eukaryotic actins, it forms bundles, rather than isolated long actin filaments, only in a restricted range of pH, and does not bind DNase-1 and phalloidin or other actin-binding toxins (15). Furthermore, this protein besides being localized in the flagellum and cytoplasm, it is also present in the nucleus and kinetoplast of *Leishmania* promastigotes where it appears to colocalize with DNA (16).

The presence of actin in the kinetoplast is intriguing as no other eukaryotic cell to our knowledge has been shown to contain this protein in the mitochondrion. To understand the functional significance of its presence in the kinetoplast, we studied the interactions of LdACT with DNA in vitro. Our studies revealed that LdACT binds and nicks DNA and converts the catenated kDNA minicircles into open form through its DNA-nicking activity. The DNA binding of this protein mainly involves electrostatic interactions between its highly diverged DNase-1-binding loop and the major groove of DNA. Furthermore, we observed that the DNA-nicking activity of LdACT is dependent of its polymeric state and ATP hydrolysis. In addition, it is observed that besides DNA, LdACT also binds bacterial type II topoisomerase and inhibits its decatenation activity. Together, these results suggest that LdACT could be involved in the kDNA remodeling process.

#### MATERIALS AND METHODS

#### Cell culture, antibodies and purification of proteins

Sf9 insect cells were maintained at 27°C as monolayer cultures in TNM-FH medium (Invitrogen). The Leishmania donovani strain (DD8) was obtained from National Institute of Immunology, New Delhi (India) and maintained at 25°C as described elsewhere (15). Antibodies to Leishmania actin (LdACT) were raised and purified by published procedures (16). Antibodies against GRP78, DNApolß and UMSBP (universal minicircle sequence-binding protein) were a kind gift from Dr E. Handman (The Walter and Eliza Hall Institute, Parkville, Victoria, Australia), Prof. Vicente Larraga (Centro de Investigaciones Biológicas, Madrid, Spain) and Prof. Joseph Shlomai (Department of Parasitology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel). respectively. Recombinant Leishmania actin (rLdACT) was purified from SF9 insect cells as reported earlier (15). β- and  $\gamma$ -Actins were purified from red blood cells and rabbit muscle acetone powder respectively as described elsewhere (17, 18).

# Subtilisin mediated cleavage of rLdACT to generate SD-rLdACT

rLdACT was cleaved with subtilisin as described earlier (19). To check the extent of cleavage, a small portion of the reaction mixture was subjected to SDS–PAGE analysis, and the remaining portion was incubated with Ni<sup>2+</sup>-NTA-agarose beads for separation of cleaved rLdACT (SD-rLdACT) from subtilisin and further purified using standard protocol. The cleaved rLdACT was checked for its polymerization ability as described earlier (15).

#### Electrophoretic mobility shift assay

Agarose gel retardation assay was performed using 400 ng of supercoiled pBR322 (New England Biolabs) or linear pBR322 DNA with rLdACT in a reaction buffer containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. After electrophoresis, gel was stained with ethidium bromide  $(0.5 \,\mu\text{g/ml})$  for 1 h, destained with TAE buffer and photographed. For Electrophoretic mobility shift assay (EMSA) on polyacrylamide gel, 100 ng of 30 bp DNA was 5'-end labeled with  $[\gamma^{-32}P]$  ATP using T4 polynucleotide kinase (NEB). The labeled fragments were purified from the unincorporated nucleotides as described earlier (20). Different concentrations of rLdACT were mixed with  $\sim$ 5 pmol of 5'-end-labeled and purified DNA fragments in a total volume of 20 µl in 20 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 and incubated for 30 min at 25°C. The reaction mixtures were loaded onto a 5% non-denaturing polyacrylamide gel (acrylamide: bis-acrylamide, 29:1). The complexes were separated at 100 V for 3 h in  $0.25 \times TBE$ . The gel was dried under vacuum and autoradiographed.

# Relaxation of negatively supercoiled plasmid DNA and kDNA decatenation

DNA-relaxation assay was performed with negatively supercoiled pBR322 plasmid DNA in the 20 µl reaction buffer containing 10 mM Tris-HCl, 0.1 mM EDTA and 2 mM ATP, pH 7.5, as described earlier (21). Decatenation of kDNA (100 ng) (Topogen, source: Crithidia fasciculata), in presence of rLdACT or SD-rLdACT was carried out under buffer conditions similar to that used in the relaxation assay. The reaction was terminated and the samples were loaded on 1% agarose gels and electrophoresed in TAE buffer at 5 V/ cm for 2.5 h. The gels were stained with ethidium bromide  $(0.5 \,\mu\text{g/ml})$ , destained and photographed. For kDNA decatenation reactions with polymeric rLdACT, polymerization of monomeric rLdACT was carried out in presence of 2mM ATP and 2mM MgCl<sub>2</sub> for 30min prior to the addition of kDNA. To ensure complete polymerization, a replicate reaction in the same buffer was monitored by dynamic light scattering measurements, as reported earlier (15).

#### Chromatin immunoprecipitation analysis

Presence of LdACT in close proximity to nuclear and kDNA *in vivo* was analyzed by Chromatin

immunoprecipitation (ChIP) assay as described earlier (22), with slight modifications. Briefly, Leishmania *donovani* promastigotes were grown to log phase  $(3 \times 10^7)$ cells/ml) and 50 ml of the cultured cells were crosslinked with 1% formaldehyde (w/v) for  $15 \min$  at  $25^{\circ}$ C. Crosslinking was stopped by incubating the mixture with a final concentration of glycine to 125 mM for another 5 min at 25°C. Cells were washed with cold PBS and resuspended in 2 ml ChIP lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate and protease inhibitor cocktail (Sigma)]. Cells were sheared on a sonicator (Branson Digital Sonifier) with 10-s pulse at 9% amplitude followed by a 30-s pause after each pulse. The resulting lysate was checked under microscope to confirm proper shearing and then centrifuged at 12000g for 10 min at 4°C. Protein concentration of the supernatant (chromatin) was determined and for each ChIP reaction, 1.0 mg protein was used. One-tenth of the lysate was reverse-crosslinked (65°C, overnight) and DNA was isolated using silica-KI method and suspended in 100 µl Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The average genomic fragment size achieved by sonication (0.5–0.8 Kb) was analyzed by agarose gel electrophoresis. To analyze DNA associated with actin, crosslinked chromatin prepared as above was subjected to ChIP using anti-recombinant LdACT antibodies. For each ChIP reaction, 100 µl of chromatin (1.0 mg protein) was diluted to 500 µl with ChIP lysis buffer and 10 µg of anti-LdACT antibodies were added. Experiments were performed using pre-immune rabbit serum and also with an irrelevant antibody (GRP78), as negative controls. Reaction mixtures were incubated for 2h at 4°C with shaking. To it was added Protein-A Sepharose beads (5 mg) after their preblocking with  $10 \,\mu$ g/ml salmon sperm DNA and 1% (w/v) acetvlated bovine serum albumin in ChIP lysis buffer for 2 h at 25°C. The resulting mixture was incubated for another 2h at 4°C with shaking. The beads were washed two times each with ChIP lysis buffer, high salt lysis buffer (same as lysis buffer but also containing 500 mM NaCl) and Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated complexes were eluted by adding 200 µl ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10mM EDTA). The elution step was repeated once again, eluates were combined and incubated at 65°C for 5 h after adding 16 µl of NaCl (5M) to reverse crosslink DNA and protein components. The mixture was further treated with 20 µg of proteinase K. DNA was extracted with phenol-chloroform and precipitated overnight at  $-20^{\circ}$ C by adding three volumes of absolute ethanol. After centrifugation at 12000g at 4°C for 30 min, pellet was washed with 70% ethanol and resuspended in 50 µl Tris-EDTA. For each PCR reaction, 0.5 µl of input DNA and 3 µl of purified ChIP DNA were used as the template. Primers for LdPFN gene (23) were used as the marker for nuclear DNA, whereas, KP1 and KP2 primers for Leishmania donovani minicircles (24) were used as the marker for the kDNA. The PCR products were analyzed on 1% agarose gel.

#### Atomic force microscopy

An amount of 400 ng supercoiled pBR322 was mixed with 1.0 µM of rLdACT in 20 µl of Tris-Cl, pH 8.0 containing 2mM ATP and incubated at 25°C for 2h. It was subsequently diluted with deionized water to a final plasmid concentration of 2.5 ng/µl and 1 mM MgCl<sub>2</sub> was added for better visualization of DNA. Totally 2 µl of this mixture was deposited on freshly cleaved mica and allowed to stand for 2 min at 25°C. It was then rinsed with deionized water (0.2-0.5 ml), air-dried and imaged in air. For imaging kDNA treated with rLdACT, 150 ng of kDNA was incubated with 2 µM of rLdACT under the similar conditions. Total 1 mM MgCl<sub>2</sub> was added to this mixture and used directly for imaging as mentioned above. Supercoiled pBR322 and untreated kDNA were also imaged with the same method except for the addition of protein. Imaging was carried out with 5500 scanning probe microscope (Agilent Technologies, Inc., AZ). Images were obtained in AAC mode with 225 µm long cantilevers that have resonance frequency of around 75 kHz and force constant of 2.8 N/m. Scan speed used was 1 line/s. Minimum image processing (first-order flattening and brightness contrast) was employed.

## HADDOCK docking

Leishmania donovani actin was docked to the DNA using the program HADDOCK 2.1 (25,26). The starting structures for the docking were a B-form model of the double-helix DNA fragment (5'- GCGGCCGCATGGC TGACAACGAGCAGAGCT-3') constructed with the 3DNA package (27) and the average model of LdACT after molecular dynamic simulations as reported previously (15). Active and passive residues for the protein were chosen based on DP-Bind server results (28,29) and solvent accessibility (>50%) was determined by the Naccess Program (25,26). A 2-Å distance was used to define the ambiguous interaction restraints (AIRs). Since the binding of actin to the DNA was found to be non-specific experimentally, therefore, for the DNA fragment, all the 30 bases were selected as 'active' and none of the base was selected as 'passive'. For LdACT AIRs were defined between all atoms of all active residues (Pro-39, Lys- 40, Met-42 and Met-46) and all atoms of all active DNA bases. Passive residues of the protein were defined as the solvent accessible surface neighbors of active residues (Gly-37, Arg-38, Asn-41, Gln-43, Gly-47, Ser-48, Ala-49, Asp-50, Lys-51, Thr-52, Val-53, Tyr-54, Gly-64 and Val-65). Additional restraints to maintain base planarity, Watson Crick bonds were introduced for the DNA. During the rigid body energy minimization, 1000 structures were calculated and the 200 best solutions based on the intermolecular energy were used for the semi-flexible, simulated annealing followed by an explicit water refinement. The solutions were clustered using a cutoff of 7.5-Å r.m.s.d. based on the pairwise backbone r.m.s.d. matrix. The best 10 structures (r.m.s.d. 0.814 Å over backbone atoms) of lowest analyzed energy cluster were using standard HADDOCK protocols and were used to represent a model of the complex. Intermolecular contacts between

LdACT and DNA nucleotides were analyzed using standard HADDOCK analysis scripts. A 3.9Å heavy-atoms distance cut-off was used for hydrophobic contacts and a 2.7Å proton–acceptor distance cut-off for hydrogen bonds.

#### RESULTS

# LdACT is associated with the chromatin and kDNA network in *Leishmania* and binds with DNA under *in vitro* conditions

Our earlier studies using immunofluorescence and immunoelectron microscopy have suggested the presence of LdACT in the nucleus and kinetoplast of Leishmania promastigotes (16). To further examine this finding, we have now analyzed the association of LdACT with chromatin and kDNA using chromatin immunoprecipitation (ChIP) assay (Figure 1A). These experiments clearly revealed the association of this protein with the chromatin and kDNA, respectively, in the nucleus and kinetoplast. The observed association of LdACT with kDNA disc raised the possibility that LdACT might directly bind DNA. We therefore analyzed the binding of recombinant Leishmania actin (rLdACT) with linearized and supercoiled DNA (scDNA) using the gel-shift assay (Figure 1B). Whereas rLdACT was observed to bind both the supercoiled and linearized DNA in a protein concentration dependent manner and converted the scDNA into its relaxed form, no such binding of  $\beta$ - or  $\gamma$ -actin with scDNA was seen in identical conditions. To further, explore whether the rLdACT binding with DNA was sequence specific, we analyzed the binding of rLdACT with <sup>32</sup>P-end labeled DNA probes by electrophoretic mobility shift assay (EMSA). The rLdACT binding with DNA was found to be not very specific to its nucleotide sequence, as rLdACT could readily bind DNA probes that were >30 nts long (Figure 1C).

To further confirm that rLdACT does have an ability to relax scDNA upon its interaction, a comparative analysis of scDNA relaxation was carried out separately with rLdACT and Topoisomerase I (Figure 2A). This analysis revealed that scDNA was indeed converted into its relaxed form upon its interaction with rLdACT. Furthermore, to establish the specificity of the scDNA-relaxation activity of rLdACT, we incubated rLdACT with scDNA in the presence of anti-LdACT antibodies (Figure 2B). As expected, rLdACT alone converted almost the entire amount of scDNA into its relaxed and linearized intermediate forms, but this activity was almost completely abolished by anti-LdACT antibodies. Furthermore, to rule out that the observed scDNA relaxation and linearization activities were specific to LdACT and were not caused by any nuclease contamination in the rLdACT preparation (15), we incubated scDNA with DNase-1 with or without rLdACT plus EDTA (Figure 2C). While scDNA was completely degraded by DNase-1 in the absence of EDTA, only relaxation of scDNA was observed when it was incubated with DNase-1 plus rLdACT and EDTA.



Figure 1. (A) ChIP analysis using anti-rLdACT antibodies showed the in vivo association of LdACT with chromatin and kDNA network. (a) and (b) are the agarose gels of PCR products after ChIP assay, showing the association of LdACT with nuclear DNA and kDNA, respectively. Lanes are marked on the top with their respective antibodies used in the ChIP assay and arrows indicated the genes amplified after pull down. An irrelevant, non-DNA associating antibody, GRP78, was used as a negative control, whereas, antibodies against DNA  $\text{pol}\beta,$ and UMSBP (universal minicircle sequence-binding protein), were used as positive controls for nuclear DNA and kDNA respectively. LdPFN, Leishmania profilin; NM12/17, specific minicircle primers. (B) Agarose gel shift assay of supercoiled and linearized pBR322 (400 ng each) in the presence of rLdACT (0.5 &  $2.0 \,\mu$ M), and  $\beta$ - and  $\gamma$ -actins (0.5 & 2.0  $\mu$ M) as indicated on the top of the gels. Lane M, shows 1 kb DNA ladder; FI: supercoiled form, FII: relaxed form, FIII: linearized form of DNA. (C) Autoradiogram of EMSA on polyacrylamide gel of  ${}^{32}P$  end-labelled 30 bp DNA probe in the presence of increasing concentration of rLdACT (0.1–0.6  $\mu$ M).

Our earlier studies have shown that rLdACT, as compared to muscle actin, exhibits high filament dynamics which involves continuous consumption of ATP (15). We therefore investigated the requirement of ATP during the scDNA-rLdACT interactions (Figure 2D and E). The scDNA-relaxation activity of rLdACT was highly dependent of hydrolysable form of ATP. As the filamentous (polymeric) state is the functional state of actin (30), we enquired whether the observed scDNA relaxation was associated with the monomeric or polymeric state of rLdACT. To address this question, we separately incubated scDNA with monomeric and



**Figure 2.** (A) Agarose gel, showing supercoiled pBR322 DNA (400 ng) relaxation separately with rLdACT (0.5 &  $1.0 \,\mu$ M) and *E. coli* Topo I. Image presented is the negative of original gel image. (B) Agarose gel electrophoresis of supercoiled pBR322 DNA (400 ng) with rLdACT in the presence or absence of anti-rLdACT antibodies, showing specificity of nicking activity associated with rLdACT. (C) Agarose gel electrophoresis of supercoiled pBR322 DNA (400 ng) with rLdACT (0.5 &  $1.0 \,\mu$ M) in the presence or absence of DNase-1 and its inhibitor EDTA which further eliminates the possibility of DNA nicking by contaminating nuclease. (D): a and b, Agarose gel electrophoresis of supercoiled pBR322 DNA (400 ng) with rLdACT (0.5 &  $2.0 \,\mu$ M) in the presence of ATP analogs as indicated on the top of the gel. (E) Graph showing the requirement of ATP in its hydrolysable form during rLdACT mediated relaxation of supercoiled pBR322 DNA. (F) Agarose gel, showing requirement of rLdACT ( $0.5 \,\& 2.0 \,\mu$ M) in the presence of increasing concentration of NaCl, inset shows the relative % inhibition of rLdACT mediated relaxation of supercoiled pBR322 DNA in the presence of 50 mM salts having different ionization constant (Ksp) as indicated on the top of the bars. (H) Dynamic light scattering measurements of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized state of rLdACT ( $1.0 \,\mu$ M) showing insignificant effect of  $0.2 \,M$  NaCl on the polymerized stat

polymeric rLdACT and then assessed the scDNA relaxation (Figure 2F). The scDNA relaxation was affected only by the polymeric and not by the monomeric rLdACT.

LdACT possesses  $\sim$ 70% amino acid sequence identity with other actins (16) and most of the diverse amino acid residues that are present in its structure are located on the

solvent-exposed surface, which differentiates it from other actins in terms of its surface charge distribution (15). As the majority of the amino acid residues present on the LdACT surface are polar in nature, binding of rLdACT with scDNA, to a major extent, may involve electrostatic interactions. To test this possibility, we analyzed the rLdACT-scDNA interactions in the presence of increasing concentrations of NaCl (Figure 2G). The extent of scDNA relaxation decreased with increasing salt concentration, indicating a major role of electrostatic interactions in this process. To further confirm the involvement of electrostatic interactions between scDNA and rLdACT, similar experiments were carried out in the presence of salts having different ionization constants, viz: CuCl and PbCl<sub>2</sub>. The inhibition of scDNA-relaxation activity associated with rLdACT was found to be greatly influenced by the ionization constant of the salt used (inset, Figure 2G). Furthermore, to check whether the increasing NaCl concentration in the scDNA-rLdACT reaction mixture affects the rLdACT polymerization status, we separately monitored the effect of NaCl on rLdACT polymerization by dynamic light scattering. rLdACT polymerization status remained unaffected under these conditions (Figure 2H).

## LdACT converts catenated kDNA minicircles into open form through its DNA-nicking activity

To analyze the functional interaction of LdACT with kDNA, we incubated kDNA with rLdACT and

observed the time dependent effect of rLdACT on kDNA network. Interestingly, due to the nicking activity associated with rLdACT, almost entire amount of catenated kDNA was converted into the major nicked form along with minor concatenated minicircle species (Figure 3A). Furthermore, to examine the specific nicking of kDNA with rLdACT, we incubated kDNA with rLdACT in the absence and presence of anti-LdACT antibodies (Figure 3B). The presence of anti-LdACT antibodies in the kDNA-rLdACT reaction mixture completely inhibited the kDNA-nicking activity of the rLdACT. Furthermore, as observed with scDNA, the kDNA-nicking activity of rLdACT was dependent of polymeric status of rLdACT and required the hydrolysable form of ATP (Figure 3E and F). To further confirm the generation of nicked DNA upon decatenation of kDNA by rLdACT, we analyzed the interactions of rLdACT with kDNA by atomic force microscopy (AFM) (Figure 4). This analysis clearly revealed the nicking of kDNA by rLdACT which is different from type II topoisomerase mediated decatenation of kDNA (31).



**Figure 3.** (A) Agarose gel (0.5%), showing the time dependent nicking of kDNA by rLdACT ( $4.0\,\mu$ M) which revealed the existence of major nicked DNA and minor concatenated minicircle species. (B) Agarose gel, showing rLdACT mediated decatenation of the kDNA network in the presence or absence of anti-rLdACT antibodies. DM, decatenated kDNA marker (Topogen). (C) Agarose gel (1.0%), showing rLdACT mediated decatenation of kDNA network with rLdACT in the presence or absence of DNase-1 and its inhibitor EDTA, which completely rules out the possibility of DNA nicking by some contaminating nuclease. (D) Agarose gel (1.0%), showing requirement of rLdACT in its polymeric state for its kDNA decatenation activity. (E): (a), Agarose gel (1.0%), showing requirement of ATP in the rLdACT mediated kDNA decatenation process. (b), Graph, showing ATP dependence of non-hydrolysable analogs of ATP. (b) Graph, showing relative inhibition of rLdACT mediated decatenation of kDNA network in the presence of non-hydrolysable ATP analogs when plotted with the increasing concentration of rLdACT.



**Figure 4.** AFM of kDNA carried out separately in the absence (control) or presence of rLdACT, showing the decatenation of kDNA with rLdACT. Panel a and b, control kDNA, arrows indicate catenated kDNA. Panel c and d, kDNA with rLdACT, arrowheads indicate decatenated nicked kDNA. (Scale bar: 500 nm).

# Diverged DNase-1-binding loop of LdACT is critical for its DNA relaxation and nicking activities

Although there is a high sequence identity between LdACT and other eukaryotic actins, a few regions are diverged which are localized on its surface. It has been predicted that these diverged regions could primarily be responsible for its unconventional behavior (15). This led us to identify the possible region(s) that might mainly be involved in binding of rLdACT with DNA. To this end, we first analyzed the protein sequence of LdACT for DNA binding, using DP-Bind server. We observed that one major DNA-binding stretch of amino acid residues was located in the DNase-1-binding (DB) loop (amino acid residues 40-53) of LdACT (Figure 5A and B), which was highly diverged, compared to other eukaryotic actins (16). Earlier studies have shown that actin monomers with bound ADP adopt  $\alpha$ -helical conformation in the DB loop region, whereas this region is disordered in ATP bound actin (32), suggesting a gain of conformation in the DB loop after ATP hydrolysis. If the DB loop does in fact undergo structural rearrangement on ATP hydrolysis, it is far more likely to be important in the polymeric form rather than the monomeric form of LdACT. This was consistent with our observations that rLdACT exhibited scDNA relaxation and kDNA decatenation activities only in its polymeric form. To further examine this point, we carried out docking of average simulated model of LdACT with non-specific 30 bp DNA sequence (as used in EMSA), by using HADDOCK protocols (25,26). It clearly revealed an interaction between the DB loop of LdACT and the DNA major groove (Figure 5C), where many residues participated in the hydrogen bonding (Figure 5D), and a



Figure 5. Computational docking of average simulated model of LdACT with DNA showed the interaction of the diverged DB-loop of LdACT with the major groove of DNA. (A) Sequence alignment of LdACT with other actins showed the conserved nuclear export signals (NES-1 and NES-2) and the diverged DB-loop predicted to be involved in the DNA binding, by DP-Bind server. (B) Energy minimized average simulated model of LdACT showing positions of NES-1, NES-2 (red) and the diverged stretches of amino acid sequences (yellow) including the sequence that fall in DB loop (blue). (C) Docking of LdACT (orange) with DNA (green) using HADDOCK protocols. (D) Amino acid residues of the DB loop of LdACT (yellow) showing hydrogen bonding interactions with the nucleotides (green) of DNA.

few showed hydrophobic interactions with the bases present in the major groove (Supplementary Table S1). This was in agreement with our finding that the scDNA-relaxation activity of rLdACT was decreased in the presence of increasing salt concentrations (Figure 1E). Moreover, energy calculations of LdACT– DNA complex after docking revealed that the resulting structure was thermodynamically stable (Supplementary Table S2).

It has earlier been shown that subtilisin (a non-specific protease from *Bacillus subtilis*) when used in 1:1500 (subtilisin to actin) mass ratio, specifically cleaves the DB loop of actin; however, after cleavage, the entire actin structure remains largely intact (Figure 6A and B) with specific disruption of the DB loop structure (19). Therefore, to determine the role of the DB loop structure, we digested rLdACT with subtilisin and then analyzed the DNA-relaxation activity and kDNA-nicking activities of DB loop cleaved actin (SD-rLdACT). It was observed that subtilisin when used in rLdACT: subtilisin mass ratio of 1000:1, it digested almost the entire amount of rLdACT within 40–50 min [Figure 6A(a)]. Analysis of the digested material showed that the cleavage of rLdACT occurred only at its N-terminus (45–53 amino acids)



Figure 6. (A) Subtilisin mediated disruption of DB loop structure of rLdACT. a, SDS–PAGE analysis of purified rLdACT and SD-rLdACT after coomassie staining; b, western blot analysis of purified rLdACT and SD-rLdACT using anti-His6 antibodies which showed the cleavage from the N-terminus, amino acid sequences, 45–53 of rLdACT; and c, western blot analysis of purified rLdACT and SD-rLdACT using anti-rLdACT antibodies that exclude the possibility of any contamination of rLdACT in the purified SD-rLdACT fraction. (B) Model showing disruption of DB loop structure after subtilisin digestion of LdACT. (C) Agarose gel (1.0%), showing kDNA nicking with the increasing concentrations of rLdACT (0.5–2.0  $\mu$ M) and SD-rLdACT (0.5–2.0  $\mu$ M) as indicated on the top of the gel. (D) Bar graph, representing relative nicking of kDNA network in the presence of rLdACT and SD-rLdACT.

[Figure 6A(b and c)] and the kDNA-nicking activity of rLdACT were considerably reduced after subtilisin digestion (Figure 6C and D).

## LdACT physically and functionally interacts with *Escherichia coli* type II topoisomerase

Several topoisomerases together with a large number of accessory proteins are involved in kDNA network organization (4). As rLdACT exhibited kDNA-nicking activity, it was possible that its presence in the kinetoplast might influence the activity of DNA topoisomerases associated with this organelle (33,34). To test this possibility, we analyzed the decatenation of kDNA by E. coli type II topoisomerase (Topo II) in the presence and absence of rLdACT. Decatenation of kDNA by Topo II in the absence of rLdACT yielded two major forms of minicircles, viz; the nicked open circular (OC) and relaxed closed circular (CC), however, in the presence of rLdACT only nicked form persisted. The intensity of the nicked band was similar to that observed with rLdACT alone (Figure 7A), suggesting a highly regulated kDNA decatenation by Topo II in the presence of rLdACT. To examine whether this influence of rLdACT on Topo II activity was due to its physical interaction with this enzyme, we incubated rLdACT separately with E. coli Topos I and II under in vitro conditions (Figure 7B). It was observed that only Topo II specifically interacted with



Figure 7. rLdACT inhibits the kDNA decatenation activity of E. coli type II topoisomerase and physically interacts with this enzyme. (A) Agarose gel (1.0%), showing nicking of kDNA in the presence or absence of rLdACT as indicated on the top of the gel. (B) SDS-PAGE showing binding of rLdACT specifically with topoisomerase-II assessed by the pulldown assay using anti-rLdACT antibodies. For these experiments, equimolar amounts of rLdACT and Topo II or Topo I were incubated in 10 mM Tris-Cl buffer (pH 7.5) containing 0.1 mM dithiothretol for 30 min. It was mixed with anti-rLdACT antibodies for 1h and then with Protein A-Sepharose beads for 15 min. The mixture was centrifuged and the beads were washed two times and proteins absorbed on beads were eluted by SDS-PAGE sample buffer and subjected to analysis. Lanes 1-3 are the purified proteins used during the pull down experiment; Lane 1, rLdACT (43 kDa); Lane 2, Topo II (heterotetramer made up of 2 gyrA (97 kDa) subunits and 2 gyrB (90 kDa) subunits); Lane 3, Topo I (holoenzyme 97 kDa). Lanes 4-11 are the proteins after pulldown using anti-LdACT antibodies. P, proteins on protein A-sepharose beads; S, unbound proteins in the supernatant. The arrow Ab, marks the antibody band.

rLdACT but no such interaction of rLdACT was seen with Topo I under identical conditions.

#### DISCUSSION

The present study reveals that LdACT, unlike other actins, readily binds DNA primarily through electrostatic interactions between its highly diverged DNase-1-binding region and the nucleotides that are located in the DNA major groove, and is associated with the kDNA network in the kinetoplast. It further shows that these LdACT-DNA interactions result in relaxation of scDNA or conversion of catenated kDNA minicircles into their open form through its DNA-nicking activity, which is dependent on its polymeric state and ATP hydrolysis. Also, it shows that LdACT specifically binds bacterial type II topoisomerase and restricts its enzymatic activity. These results, for the first time, reveal the association of a cytoskeleton protein, like actin, with the mitochondrial genome of a kinetoplastid and highlight the functional significance of this association during kDNA network remodeling.

kDNA is a complex network of the mitochondrial genome consisting of minicircles and maxicircles of DNA. A remarkable degree of cooperation is observed between the maxicircles and minicircles during kDNA expression: whereas the maxicircles transcribe unprocessed mRNAs for several respiratory enzymes. minicircles transcribe the sequences that are required to edit these unprocessed mRNAs into the functional form (4). Unique to the kinetoplastid mitochondria, many proteins have been predicted to be involved in kDNA organization including unknown sets of cytoskeleton proteins (4,35). Results presented here clearly demonstrate the presence of actin in the *Leishmania* kinetoplast where it is associated with kDNA. Earlier studies have shown that actin is an integral component of the ATP dependent chromatin remodeling complexes in the nucleus where it is involved in a variety of processes such as transcription, RNA transport and chromatin remodeling (36). The precise functions of various proteins that are involved in the chromatin remodeling are, however, not fully (4,35) understood, but under in vitro conditions, some of these proteins have been characterized to disrupt, exchange and slide nucleosomes, some influence DNA supercoiling and others have been shown to be associated with acetylases and deacetylases (36-39). The DNA-relaxation activity of LdACT together with its presence in the nucleus and kinetoplast observed in the present study indicate that this protein perhaps is an important component of one or more of the protein complexes that are required for chromatin or kDNA remodeling in Leishmania. Furthermore, besides the relaxed form, we have also observed existence of the intermediate linearized form of DNA during the LdACT-mediated scDNA-relaxation process, which suggests that LdACT possesses a DNAnicking activity. Among topoisomerases, such a characteristic relaxation activity has earlier been shown to be associated with the alpha subunit of human type-II topoisomerases (40).

It has earlier been observed that recombinase proteins, such as RecA and RAD51, form ATP-dependent nucleoprotein filaments around the homologous duplex DNA and catalyze a strand exchange during repair of double strand breaks in DNA. This is followed by ATP-dependent disassembly of these filaments that occurs from their ends (41), suggesting a high dynamics of nucleoprotein filaments during the DNA damage-repair process. As ATP hydrolysis is considerably increased during the LdACT-mediated catenated kDNA minicircle nicking and opening process, we infer that perhaps a highly dynamic assembly of LdACT filaments occurs on the kDNA surface which catalyzes the decatenation process.

Replication of the mitochondrial genome in kinetoplastids occurs by a unique mechanism that has not been observed in any other organism to date. In non-replicating Leishmania kDNA network, each minicircle is linked to about three neighboring minicircles (three valence state). But as replication proceeds, the number of minicircles grows and the minicircle valence rises from three to six due to the physical constraints imposed by the limited space available to the network in a specialized region of the mitochondrion. However, as the cell proceeds through the growth phase, the available space to the network increases and the minicircle valence then drops back from six to three (6). The factors that constrain the network volume in the mitochondrial matrix during the replication process are, however, not known, but a role of mitochondrial membrane or some unknown cytoskeleton structure has been speculated (4). Here, we suggest that LdACT filaments could fulfill this role.

Increase in the minicircle valence from three to six during kDNA replication in the S-phase and its dropping back again to three during the G2-phase of the cell cycle occurs in a process termed as kDNA remodeling

(6). For kDNA replication to occur, each minicircle must be individually delinked from the network structure in order to freely replicate, and after completion of its replication, the progeny minicircles must reattach back to the network. All the newly replicated minicircles contain at least one nick or gap until all the minicircles have undergone replication. As replication proceeds, the network size grows and ultimately doubles after all the minicircles have replicated. Finally, nicks and gaps in the minicircles are repaired and a scission occurs along the diameter of the doubly sized network to split it into two equal sized progeny networks. The process of maintenance of at least one nick or gap in the progeny minicircles throughout replication as well as scission of the doubly sized network has been proposed to involve a set of proteins that could restrict the activity of topoisomerase II (4). As results presented here clearly show that LdACT physically interacts with Topo II and inhibits its decatenation activity, we propose that LdACT could play a critical role in specific regulation of type II topoisomerase(s) during the above processes.

Finally, it has recently been shown that a prokaryotic homolog of actin, MreB, participates in chromosome segregation by regulating the activity of topoisomerase IV in *E. coli* (42). It has further been shown that MreB does not bind DNA but it physically and functionally interacts with Topo IV. However, LdACT, though physically and functionally interacts with bacterial type II topoisomerase, but it also binds DNA and exhibits DNA-nicking activity, a property that to our knowledge has not been observed with any other actin. These results thus reveal that LdACT is a unique form of eukaryotic actin which differs from other actins not only in terms of its filament forming, toxin and DNase-1-binding properties (15) but also in DNA binding, DNA nicking and topoisomerase II regulation properties.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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