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A novel form of actin in *Leishmania*: molecular characterisation, subcellular localisation and association with subpellicular microtubules☆

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Abstract

To study the occurrence and subcellular distribution of actin in trypanosomatid parasites, we have cloned and overexpressed *Leishmania donovani* actin gene in bacteria, purified the protein, and employed the affinity purified rabbit polyclonal anti-recombinant actin antibodies as a probe to study the organisation and subcellular distribution of actin in *Leishmania* cells. The *Leishmania* actin did not cross react with antimammalian actin antibodies but was readily recognized by the anti-*Leishmania* actin antibodies in both the promastigote and amastigote forms of the parasite. About 10^6 copies per cell of this protein (M_r 42.05 kDa) were present in the *Leishmania* promastigote. Unlike other eukaryotic actins, the oligomeric forms of *Leishmania* actin were not stained by phalloidin nor were dissociated by actin filament-disrupting agents, like Latrunculin B and Cytochalasin D. Analysis of the primary structure of this protein revealed that these unusual characteristics may be related to the presence of highly diverged amino acids in the DNase I-binding loop (amino acids 40–50) and the hydrophobic plug (amino acids 262–272) regions of *Leishmania* actin.

The subcellular distribution of actin was studied in the *Leishmania* promastigotes by employing immunoelectron and immunofluorescence microscopies. This protein was present not only in the flagella, flagellar pocket, nucleus and the kinetoplast but it was also localized on the nuclear, vacuolar and cytoplasmic face of the plasma membranes. Further, the plasma membrane-associated actin was colocalised with subpellicular microtubules, while most of the actin present in the kinetoplast colocalised with the k-DNA network.

These results clearly indicate that *Leishmania* contains a novel form of actin which may structurally and functionally differ from other eukaryotic actins. The functional significance of these observations is discussed.

Keywords: Trypanosomatids; Actin; Microtubules; k-DNA network; Association; Cellular functions

1. Introduction

Actin is a cytoskeletal protein, which besides determining the cell morphology also provides the building blocks required for cell motility, contractility and intracellular transport in eukaryotic cells [1,2]. The protein exists in monomeric as well as filamentous forms. The filaments are organised in specific arrays that form protrusive structures at the cell surface and also associate with the cytoplasmic surface of the plasma membrane to regulate the cell shape [2,3]. Also, actin has been detected in the nucleus where it has been implicated to play an important role in chromatin remodeling [4,5], and also in RNA processing, transport and other related processes [6,7]. Furthermore, the actin network functionally associates with microtubules during processes, such as cell migration, cleavage furrow placement and vesicle transport [8,9], and also with the plasma membrane regions that are engaged during endocytosis [10,11]. Due to its multifunctional roles in cell multiplication, growth and development, actin is a most abundant protein in many eukaryotic cells and is conserved from yeast to humans [2].

Leishmania parasites are protozoan organisms which cause the dreaded disease 'Kala-azar' and exist in two forms, viz. promastigotes and amastigotes, which contain microtubules as their major cytoskeletal component [12]. Although these cells have been shown to carryout some of

Abbreviations: DMEM, Dulbecco's modified eagle medium; L-act Abs, monospecific polyclonal anti-*Leishmania* actin antibodies; M-act Abs, polyclonal anti-human actin antibodies; DAPI, 4′,5-diamidino-2-phenylindole

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the cellular activities that essentially require actin [13,14], no convincing evidence to date is available on the existence of actin network in *Leishamnia* cells [12,15,16]. However, the presence of actin gene has been confirmed in the *Leishmania major* genome [17].

To examine the presence of actin in *Leishmania*, we prepared affinity purified monospecific anti-recombinant *Leishmania* actin antibodies and then by using these antibodies as a probe we analysed some molecular characteristics, subcellular localization and association with microtubules of actin in the *Leishmania* promastigotes. Our results revealed that about 10^6 copies of actin (M_r 42.05 kDa) per cell were present in form of granules, short filaments/bundles and patches in the *Leishmania* promastigotes. It is further shown that unlike conventional actins, these oligomeric forms of *Leishmania* actin were neither disrupted by the actin filament disrupting agents nor stained by phalloidin. In addition, it is demonstrated that this protein in these cells was localized not only in their cortical cytoskeleton, flagella, flagellar pocket, nucleus and kinetoplast, but also on the vacuolar and nuclear membranes, and also it is closely associated with the subpellicular microtubules and the k-DNA network. These results indicate that kinetoplastid parasites, especially *Leishmania*, contain unconventional actin which may structurally and functionally differ from other eukaryotic actins.

2. Materials and methods

2.1. Cell culture

All the *Leishmania* strains and mouse macrophage cell line (J774 A.1) and BHK21 cells were obtained from the National Institute of Immunology, New Delhi (India), and maintained in high glucose DMEM, supplemented with 10% heat-inactivated fetal bovine serum containing 40 μg/ml gentamycin, at 25 and 37 °C, respectively. Monolayers of macrophage cells on coverslips were infected with *Leishmania donovani* (DD8 strain) promastigotes following the published procedure [18].

2.2. Cloning and expression of Leishmania donovani actin

Genomic DNA from the cultured promastigotes was prepared using standard protocols [19]. Primers were designed on the basis of the *Leishmania major* actin gene sequence available from the Gene Bank (accession no. L16961). Putative actin gene of about 1.1 kb was amplified by polymerase chain reaction (PCR) using the forward primer: 5′-GGAATTCCATATGGCTGACAACGAGCAGAGCTCCATCG-3′, reverse primer: 5′-GGAATTCCATATGTCAGAAGCACTTGTTATGCACGATGCT-3′ and the total genomic DNA as the template. PCR product was cloned in the prokaryotic expression vector pET-22b (Novagen, USA) at Nde I site, sequenced by dideoxy chain termination method [20], and then overexpressed in *Escherichia coli* strain BL21 (DE3). For expression in mammalian cells (BHK21 cell line), the *Leishmania* actin gene was cloned in pCDNA3 vector (Invitrogen, USA). Transfection quality DNA of this construct was made by Qiagen Tip 100 midiprep column. BHK21 cells were transfected with pCDNA3-actin construct using Lipofectamine-Plus reagent (GIBCO-BRL, USA).

2.3. Actin sample preparation for ESI-mass spectrometry

The recombinant *E. coli* cells obtained from the induced cultures (5.0 ml) were sonicated in water (0.5 ml) with 10 pulses of 10 s each with 20 s intervals on ice. The cell lysate thus obtained was centrifuged first at 500 × *g* for 10 min to remove the cell debris and then the milky supernatant at 12000 × *g* for 5 min (4 °C) to pellet out the aggregated actin present in inclusion bodies. The pellet was washed with water for six times and then dissolved in absolute formic acid (50 μl). It was centrifuged at 12,000 × *g* and the clear supernatant was analysed by mass spectrometry.

2.4. Antibodies

The recombinant *Leishmania donovani* actin was purified by SDS–polyacrylamide gel electrophoresis [21]. Purified recombinant protein was injected into rabbits to raise the polyclonal antiserum. Anti-*Leishmania* actin antibodies were affinity purified by using recombinant actin after its purification by preparative SDS–polyacrylamide gel electrophoresis followed by immobilization on the CNBr-activated Sepharose (Amersham-Pharmacia Biotech). Antibodies against α,β-tubulins (monoclonals) and human β-actin (polyclonal and monoclonal)) were procured from ICN, USA.

2.5. Western blotting

Proteins resolved on the SDS–polyacrylamide (10%) gels were electroblotted onto the nitrocellulose membranes in Tris-glycine buffer at 50 V for 2 h [22]. After incubating the blots with primary antibodies (0.1 μg/ml) at 25 °C for 1 h followed by five washings with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, these were further incubated with HRP conjugate of secondary antibodies, washed, and developed by chemiluminescence (ECL kit, Amersham Pharmacia, USA).

2.6. Immunofluorescence microscopy

Leishmania promastigotes were fixed with 4% (w/v) paraformaldehyde in 200 mM HEPES buffer (pH 7.2) at 25 °C for 30 min, and washed two times for 5 min each with PBS containing 0.5% (w/v) glycine. The washed cells were allowed to adhere on the poly-L-lysine-coated coverslips. To analyze the *Leishmania* amastigotes, the mouse macrophage cells (J774A.1) were grown on coverslips and infected with *Leishmania* promastigotes (1:10 ratio)

at 37 °C for 1 h. The macrophage cells 24 h post infection were fixed with 4% paraformaldehyde (w/v) in PBS (pH 7.2) at 25 °C for 10 min, and washed as above.

The fixed cells were permeabilised with 0.1% (v/v) Triton X-100 and blocked with 10% (v/v) goat serum (Amersham Pharmacia) in PBS for 30 min. The blocked cells were first incubated with primary antibodies (10 μg/ml) at 4 °C and then stained with Oregon green and/or Texas red-X-tagged secondary antibodies (10 μg/ml) at 4 °C for 4 h in dark. Nuclei were stained with 4′,5-diamidino-2-phenylindole (DAPI) (5 μg/ml). Coverslips were mounted in the mounting medium (Oncogene) and images were collected using 63 × 1.4 NA Plan Apochromate lense on Zeiss LSM 510 Confocal Microscope. Green, red and blue images were collected separately with excitation at 488, 543 and 364 nm, respectively, and merged for presentation.

2.7. Immunogold electron microscopy

Leishmania promastigotes were fixed with 4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in PBS. This was followed by two more changes of fixatives and subsequent incubations for 4 h. The excess fixatives were quenched with 0.5% (w/v) glycine in PBS (pH 7.4) at 4 °C for 10 min. The fixed cells embedded in 1% agarose. The agarose-embedded blocks were dehydrated in ascending series of ethanol, impregnated in LR-White resin (Sigma) and then polymerized at 60 °C for 48 h. Ultrathin sections (80–100 nm) were cut and collected on nickel grids. Sections were first blocked by PBS (pH 7.4) containing 0.1% BSA-c (w/v) (Aurion, Germany), and then incubated for 4 h with monospecific anti-*Leishmania* actin antibodies (10 μg/ml) in the blocking buffer. After washing five times with the blocking buffer, the sections were incubated with 10 nm gold-coupled goat anti-rabbit IgG (1:10) at 37 °C for 1 h, washed with blocking buffer five times for 5 min each, contrasted with uranyl acetate and examined under FEI Philips Transmission Electron Microscope (TECNAI-12) at 80 kV. Control samples were separately incubated as above with normal rabbit IgG as well as polyclonal anti-human actin antibodies rather than monospecific polyclonal anti-*Leishmania* actin antibodies.

2.8. Latrunculin B treatment

Transfected or non-transfected BHK21 cells and *Leishmania* promastigotes were treated with Latrunculin B (5 μM) under their growth conditions for 30 min. Cells were harvested, washed, and then lysed in PBS containing 1% (v/v) Triton X-100 and protease inhibitor cocktail on ice (15 min). The lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C and the clear supernatants were carefully collected as separate soluble fractions. Insoluble pellets washed once with the above buffer and washings were added to the above soluble fractions. Equal volumes of both the soluble and insoluble fractions were analysed for actin by Western blotting and the amount of actin quantitated by densitometric analysis using the Image Master programme (Amersham-Pharmacia).

2.9. Protein copy number determination

Estimated quantities of purified recombinant actin and lysates of known number of *Leishmania* promastigotes were resolved on 10% SDS–polyacrylamide gels and electroblotted on nitrocellulose membrane. Blots were developed after primary and secondary antibody treatment by ECL kit (Amersham Pharmacia, USA) on X-ray film. The bands were analysed by densitometric analysis and the amount of actin in known number of cells was determined from the standard curve derived from purified actin. The standard curve was linear at least upto 100 ng quantity of actin.

3. Results

3.1. Molecular characterization

We cloned and over expressed the *L. donovani* actin gene in *E. coli* (Fig. 1) by using the primers that were designed on the basis of the sequence reported earlier for *L. major* actin gene [17]. The molecular identity of recombinant actin was established by sequencing a portion

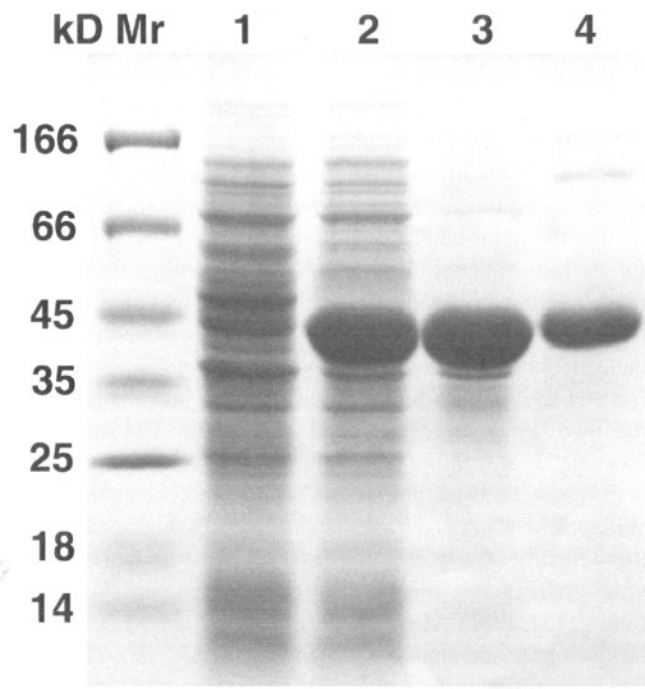

Fig. 1. Over expression of *Leishmania* actin in *E. coli*. The cell lysates were analysed by SDS–polyacryamide gel electrophoresis (PAGE) and the gels stained with Coomassie blue. M_r, molecular weight markers; lane 1, uninduced cell lysates; lane 2, induced cell lysates; lane 3, inclusion bodies; lane 4, SDS–PAGE purified recombinant *Leishmania* actin (M_r 42.05 kDa). The total protein in lanes 1–3 was 30 μg/ lane, while lane 4 contained only 10 μg protein.

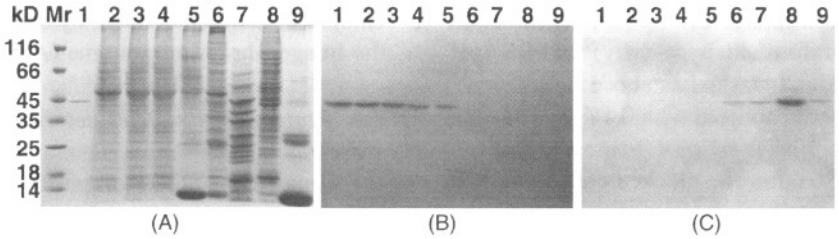

Fig. 2. Specificity of anti recombinant *Leishmania* actin antibodies. Panel A: Coomassie-blue stained SDS–PAGE (10%); panel B: Western blotting with anti-*Leishmania* actin antibodies; panel C: Western blotting with anti-human actin antibodies. M_r, molecular weight markers; lane 1, recombinant actin; lane 2, *L. donovani* promastigote lysate; lane 3, *L. major* promastigote lysate; lane 4, *L. tropica* promastigote lysate; lane 5, *T. cruzi* (blood stage) lysate which contained about 20% mouse erythrocytes as contamination; lane 6, *P. falciparum* lysate; lane 7, *S. cerevisiae* lysate; lane 8, BHK21 cells lysate; lane 9, mouse erythrocytes lysate. Lanes 2–9 contained 30 μg protein/lane whereas lane 1 contained only 0.5 μg protein.

of its N-terminal sequence (ADNEQSSIVXD) and also analyzing by mass spectrometry. The molecular mass of recombinant actin was 42.05 kDa which corresponded well with the molecular mass of eukaryotic actins [1,2]. Pure recombinant actin in monomeric form was prepared from actin aggregates by SDS–polyacrylamide gel electrophoresis [21], and used for immunization of rabbits for raising the anti-actin antisera. The immunogenicity of the recombinant *Leishmania* actin was about 100 times higher than that of the human erythrocyte actin as judged from the levels of antibody titres in the rabbits that were separately immunized with equal quantities of *Leishmania* and human erythrocyte actins under identical conditions (data not shown).

Monospecific polyclonal anti-*Leishmania* actin antibodies (L-act Abs) were prepared from the rabbit antisera and used to probe the presence of actin in *Leishmania* cells. Fig. 2 shows that L-act Abs strongly crossreacted with a protein of about 43 kDa in whole cell lysates of various *Leishmania* species as well as of *Trypanosoma cruzi*, but it failed to recognize the actin band in whole cell lysates of *Plasmodium falciparum, Saccharomyces cerevisiae*, BHK21 cells and human erythrocytes. Conversely, rabbit polyclonal anti-human β-actin antibodies (M-act Abs) readily recognized the actin band in whole cell lysates of *P. falciparum, S. cerevisiae*, BHK21 cells and human erythrocytes but it did not cross react with *Leishmania* or *Trypanosoma* actin. To further corroborate these findings, we analysed the presence of actin in *L. donovani* by immunofluorescence microscopy (Fig. 3). Whereas M-act Abs even upon using in very high concentration failed to clearly detect the presence of actin in *Leishmania* promastigotes, which is not in agreement with the earlier report [15], L-act Abs readily recognized actin in both the promastigote and amastigote forms of *L. donovani*. This protein was present largely in granular form not only in the main body of the promastigote but also in its flagella. The average number of actin molecules was about 1×10^6 copies per promastigote. Further, unlike the mammalian actin, the *Leishmania* actin was not stained by rhodamine-phalloidin which is known to specifically stain actin in its filamentous form [23].

Significant fraction of the total cell actin exists in oligomeric forms in the eukaryotic cells [1,2]. These oligomers are, however, dissociated into actin monomers by treating the cells with Latrunculin B or Cytochalasins [24], but resist their solubilisation by Triton X-100 [25]. To determine the fraction of *Leishmania* actin that exists in the oligomeric form, we therefore treated the *L. donovani* promastigotes and BHK21 cells with Latrunculin B in identical conditions, and then determined the fractions of monomeric and oligomeric forms of actin prior to and after subjecting them to the Triton X-100 solubilisation method [25]. Fig. 4A and B shows that only about 30% of the total *Leishmania* actin was insoluble in the detergent, as compared to about 45% of the total BHK 21 cell actin. Further, unlike the BHK 21 actin, the oligomeric forms of *Leishmania* actin were almost completely resistant to the Latrunculin B treatment. Similar results were also observed by treating the *Leishmania* promastigotes with Cytochalasin D (data not shown). To examine whether the reduced extent of *Leishmania* actin oligomerisation in the promastigotes is due to absence of some proteins [3,26] that assist/promote actin in forming large filaments or it is due to some structural differences between the mammalian and *Leishmania* actins, we expressed the *Leishmania* actin in BHK 21 cells and then estimated the amounts of oligomeric forms of both the BHK 21 and *Leishmania* actins in the transfected cells. Results given in Fig. 4C and D clearly indicate that the extent of oligomerisation of *Leishmania* actin did not increase even upon expressing this protein in the mammalian cells.

These results strongly indicate that *Leishmania* actin significantly differs from yeast, *Plasmodium* and human actins in terms of its properties. To examine the structural basis of this difference, we carried out the amino acid sequence alignment analysis and homology modeling (Fig. 5). *Leishmania* actin possessed 70.93, 69.14 and 69.76% amino acid identities, respectively, with yeast, *Plasmodium* and human actins. The major differences were confined to the amino acids (aa) 1–9, 40–53, 194–200, 229–240, 266–281 and 307–315, most of which were located on the surface of yeast or mammalian actins, and one of these included the amino acid residues, aa

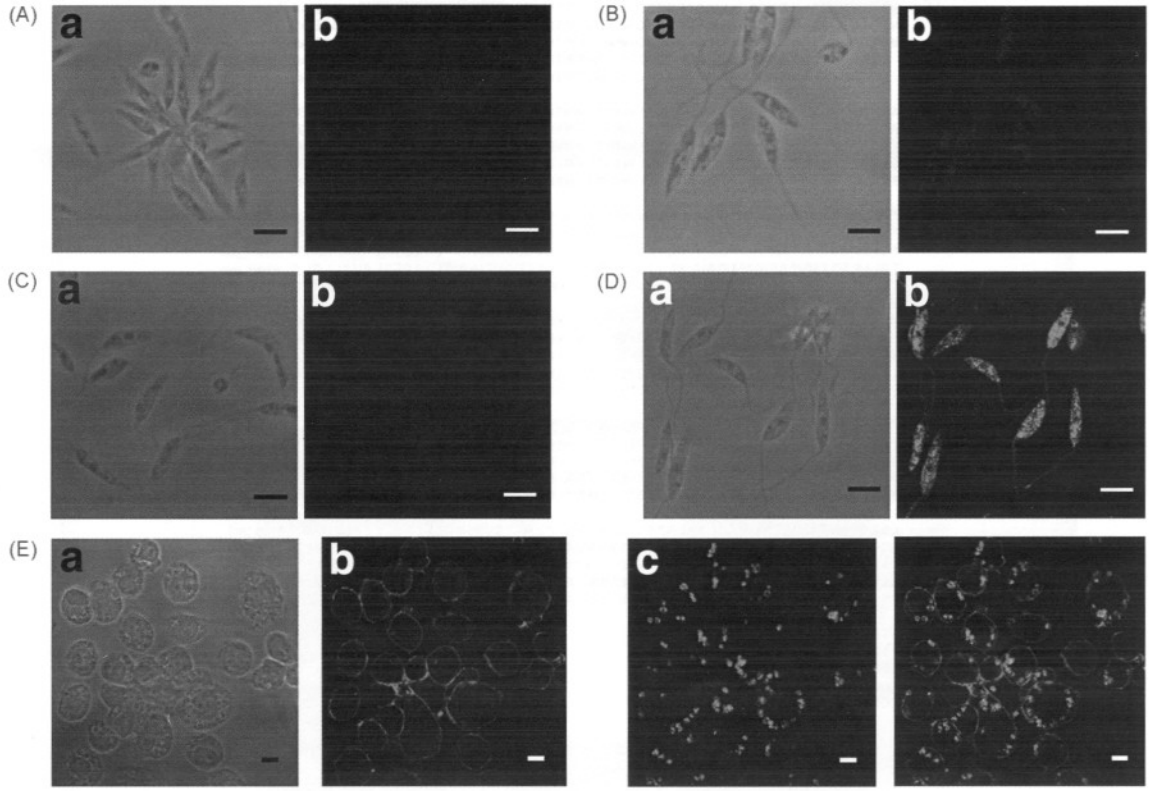

Fig. 3. Immunostaining of actin in *L. donovani* promastigotes and amastigotes with anti-*Leishmania* actin antibodies. The control cells (promastigotes) were stained with secondary antibodies (panel A: a, phase micrograph; b, immunofluorescence micrograph), anti-human actin antibodies (panel B: a, phase micrograph; b, immunofluorescence micrograph) and rhodamine-phalloidin (panel C: a, phase micrograph; b, fluorescence micrograph). Panel D: phase (a) and immunofluorescence (b) micrographs of *Leishmania* promastigotes stained with anti-*Leishmania* actin antibodies. Panel E: *L. donovani* infected mouse macrophages (J774A.1) doubly stained with rhodamine-phalloidin and anti-*Leishmania* actin antibodies. A, phase; b, rhodamine-phalloidin; c, anti-*Leishmania* actin antibodies; d, merge of b and c. Bar, 5 μm.

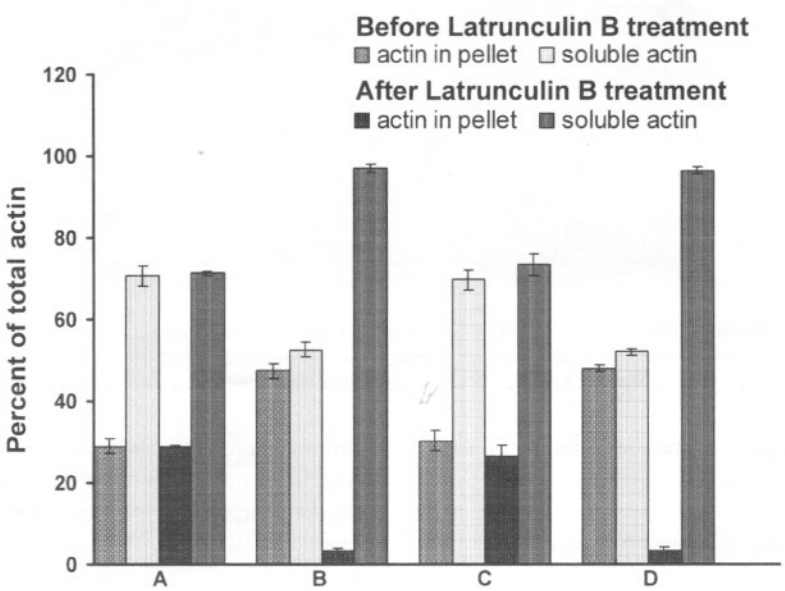

Fig. 4. Effect of Latrunculin B on Triton X-100-insoluble fraction of *Leishmania* and mammalian actins. (A) *Leishmania* actin in *L. donovani* promastigotes; (B) mammalian actin in BHK21 cells; (C) *Leishmania* actin in BHK21 cells transfected with *Leishmania* actin gene; (D) mammalian actin in BHK21 cells transfected with *Leishmania* actin gene. Values shown are means of three independent experiments ± standard deviation. The standard deviation was calculated by using Student's '*t*' test.

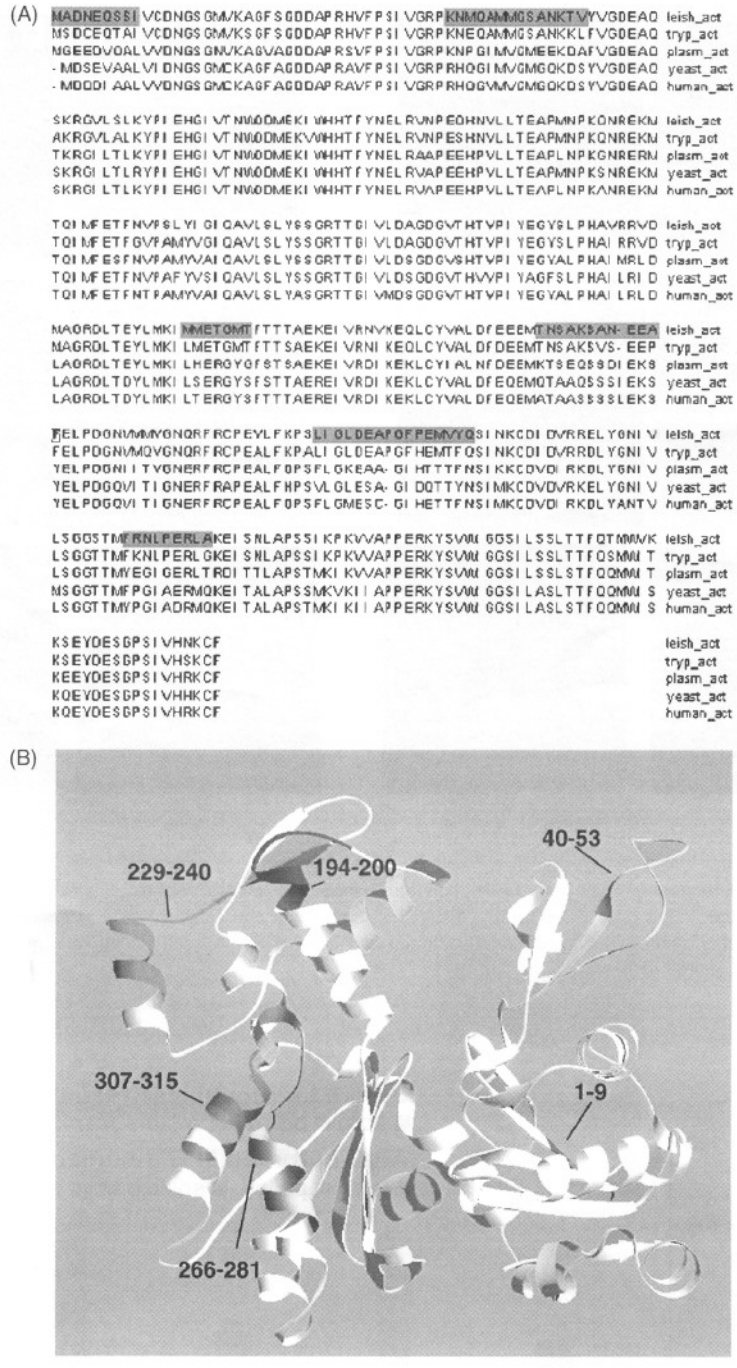

Fig. 5. (A) Amino acid sequence alignment of *Leishmania* (leish), *Trypanosoma* (tryp), *Plasmodium* (plasm), Yeast and human actins. The shaded regions denote the divergent sequences in *Leishmania* actin. *L. donovani* actin gene sequence submitted to EMBL Gene Bank (AY637036). (B) G-actin model showing the aminoacid sequences that are divergent in *Leishmania* actin. Homology modelling was carried out as in [38].

42–46, that constitute the major self association sites during the actin filament formation [2].

3.2. Subcellular localization and association with microtubules

Subcellular localization of *Leishmania* actin was studied by the immunoelectron and immunofluorescence microcopies. Fig. 6 shows that this protein in *Leishmania* promastigotes was present not only in the flagellar pocket, flagella, kinetoplast, nucleus and cytoplasm but also on the nuclear, vacular and cytoplasmic face of plasma membranes. Actin in flagella was localized predominantly on its surface rather than the interior. Likewise, in the kinetoplast, this protein seemed to largely localize in the k-DNA network. Similarly, in the cortical region, it appeared to localize mainly on the microtubular network that constitute the cortical cytoskeleton. This apart, short filament-like

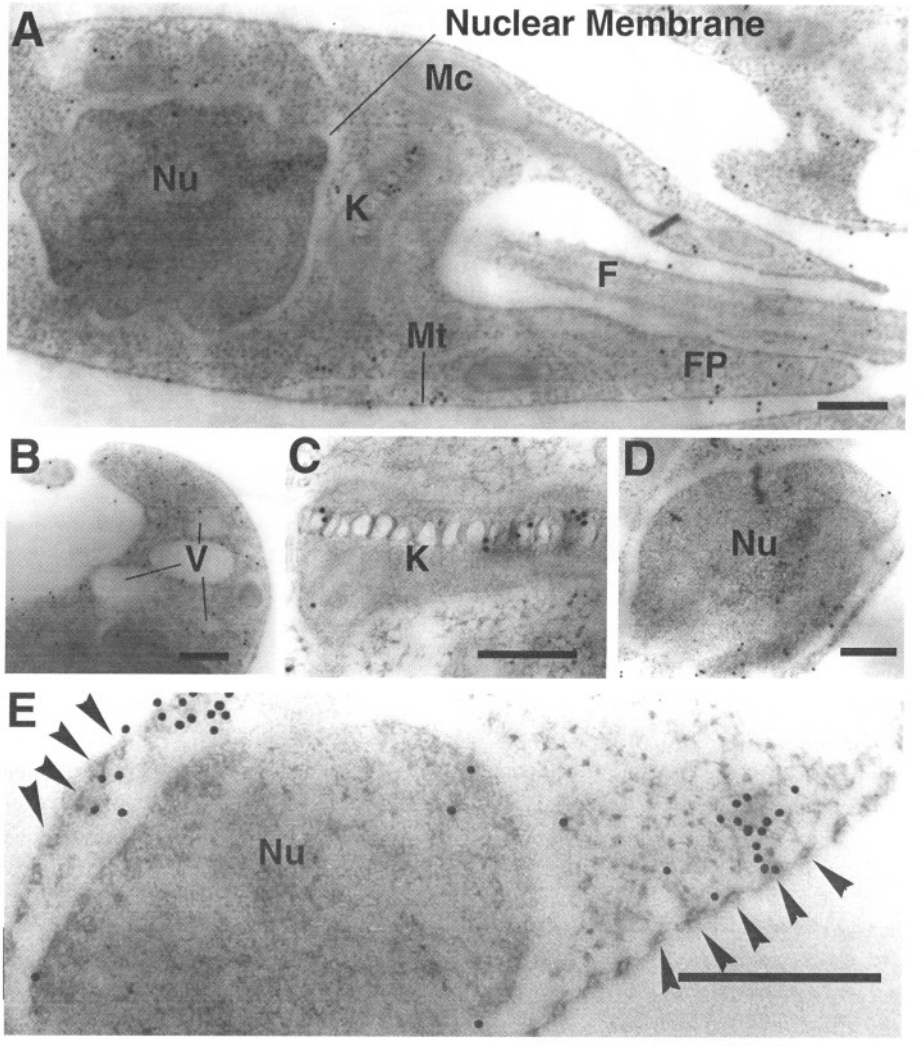

Fig. 6. Electron microscopy of immunogold-labeled actin in *Leishmania* promastigotes. Localization of actin on as well as in close vicinity of subpellicular microtubules (Mt), flagella (F), flagellar pocket (FP), kinetoplast (K), nucleus (Nu), cytoplasm and nuclear membrane is clearly visible in panel A. In addition, presence of actin on membranes of vacuoles (V) may also be noticed in panel B. Associations of actin with kDNA network, nuclear membrane and subpellicular microtubules may clearly be seen in panels C–E, respectively. The arrowheads in panel E mark the microtubules. The control samples were incubated with normal rabbit IgG as well as polyclonal anti-human actin antibodies rather than the anti-*Leishmania* actin antibodies. At least 120–150 fields were examined and most of them were found to have similar actin distribution patterns. Mc is mitochondria. Bar, 200 nm.

structures of actin were seen in cortical as well as other regions.

To further confirm the above findings, we examined the actin distribution in the *Leishmania* promastigotes cytoskeletons that were largely devoid of the cytoplasm but contained the nucleus and kinetoplast by employing immunofluorescence microscopy. The cytoskeletons were prepared by treating the cells with varying amounts of NP-40 or Triton X-100 (Fig. 7) as described earlier for preparation of *Trypanosoma* cytoskeletons [27,28]. Whereas treatment of the promastigotes with 1% Triton X-100 resulted in loss of structural integrity of the nucleus and kinetoplast, use of 0.1–0.5% NP-40 yielded the cytoskeletons that contained these organelles largely in their intact form, as judged by light fluorescence microscopy (data not shown). Therefore, only the cytoskeletons that were obtained by the 0.5% NP-40 treatment were used for the immunofluorescence studies to analyse the presence of actin in the nucleus and kinetoplast. Results shown in Fig. 8 clearly indicate that besides being localized in the posterior end, flagella, cortical cytoskeleton and flagellar pocket, actin was present also within the nucleus and kinetoplast in form of short filaments, bundles and beaded structures. Further, at least a part of the actin that was localized in the nucleus and kinetoplast appeared to associate with DNA in both the organelles. This apart, most of the kinetoplast surface was covered by the actin network which seemed to serve as an anchor between the kinetoplast and the cell surface.

It would seem that actin in the *Leishmania* promastigotes associates with cortical microtubules. To further examine this possibility, we studied both the intact promastigotes and their cytoskeletons, obtained by the1% Triton X-100 treat-

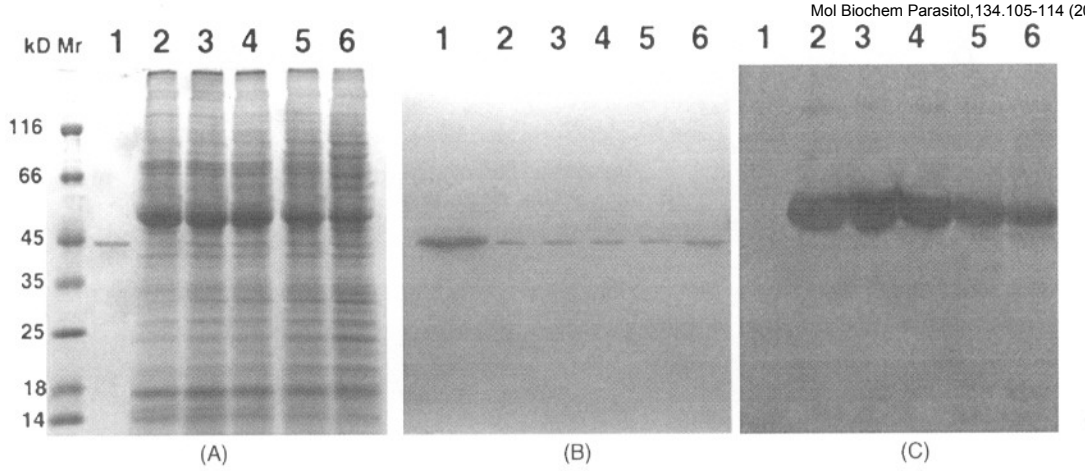

Fig. 7. Preparation of *Leishmania* promastigote cytoskeletons. The cells were treated with Triton X-100 (1%) or varying concentrations of NP-40 for 5 min on ice and the lysates centrifuged at 12,000 × g (4 °C) for 10 min. The pellets were washed with buffer and then analysed by SDS–PAGE. Panel A, Coommassie blue stained SDS–PAGE; panel B, Western blotting with anti-*Leishmania* actin antibody; Panel C, Western blotting with anti-α and β-tubulin. M_r, molecular weight markers; lane 1, recombinant actin (0.5 μg); lane 2, 1% Triton X-100; lane 3, 0.5% NP-40; lane 4, 0.1% NP-40; lane 5, 0.05% NP-40; lane 6, 0.025% NP-40; lanes 2–6 contained 30 μg total protein.

ment, by immunofluorescence microscopy. Fig. 9 shows that cortical actin in *Leishmania* is colocalized with microtubules in both the whole cells and cytoskeletons, confirming its association with microtubules, at least in the cortical region.

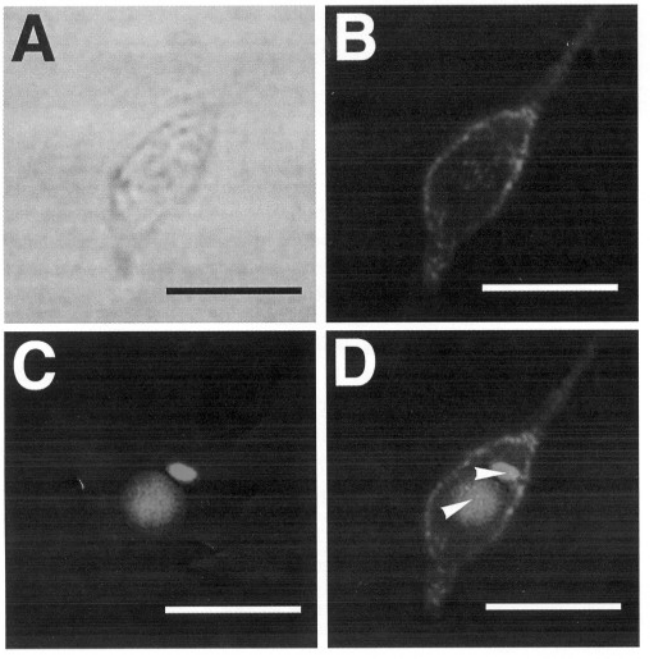

Fig. 8. Immunofluorescence microscopy of *Leishmania* promastigote cytoskeletons prepared by treating the cells with 0. 5% NP-40. The cytoskeletons were doubly stained with anti-*Leishmania* actin antibodies and DAPI. Panel A, phase; panel B, *Leishmania* actin; panel C, DNA in nucleus and kinetoplast; panel D, merge of B and C. Arrowheads indicate the regions where association of actin with DNA is clearly visible. Bar, 5 μm.

4. Discussion

Microtubules and actin microfilaments are the two major components of the eukaryotic cell cytoskeleton. While the microtubule network has been well characterized in kinetoplastid parasites [12], virtually nothing is known about the existence and functions of the actin cytoskeleton in these organisms. The present study, for the first time, reports that about 10^6 actin copies per cell are present in the *Leishmania* promastigote, and shows that *Leishmania* actin significantly differs from other eukaryotic actins, especially plasmodia, yeast and mammalian actins, in terms of its immunogenic and filament-forming properties. Unlike mammalian or yeast actins [1,2], *Leishmania* actin does not form arrays nor are its oligomeric forms stained by phalloidin or dissociated by Latrunculin B or Cytochalasin D. However, besides its localization in flagella, flagellar pocket and kinetoplast, it is, like in other eukaryotic cells [1,2,4,5], also present in the cortical cytoskeleton and nucleus.

There are atleast six different isoforms of actin in mammals; two cytoplasmic actins, β ad γ, and four muscle actins. The major differences between the mammalian actins and *Leishmania* actin exist in the amino acid sequences of which three sequences, viz. aa 40–53, aa 194–200 and aa 266–281, include partially or wholly the sites that participate in subunit–subunit contacts during the actin oligomerization to form filamentous actin [29,30]. Further, besides being important for the subunit-subunit contacts, the amino acid residues 40–53 also include the DNase I binding loop [29,30]. In addition, mutation ($L_{266}D$) in the aa 262–272 region, which constitutes the hydrophobic plug [29,30], in yeast actin has been reported to disrupt the hydrophobic interactions, resulting in inhibition of actin polymerisation at low temperature [31]. We therefore conclude that *Leishma-*

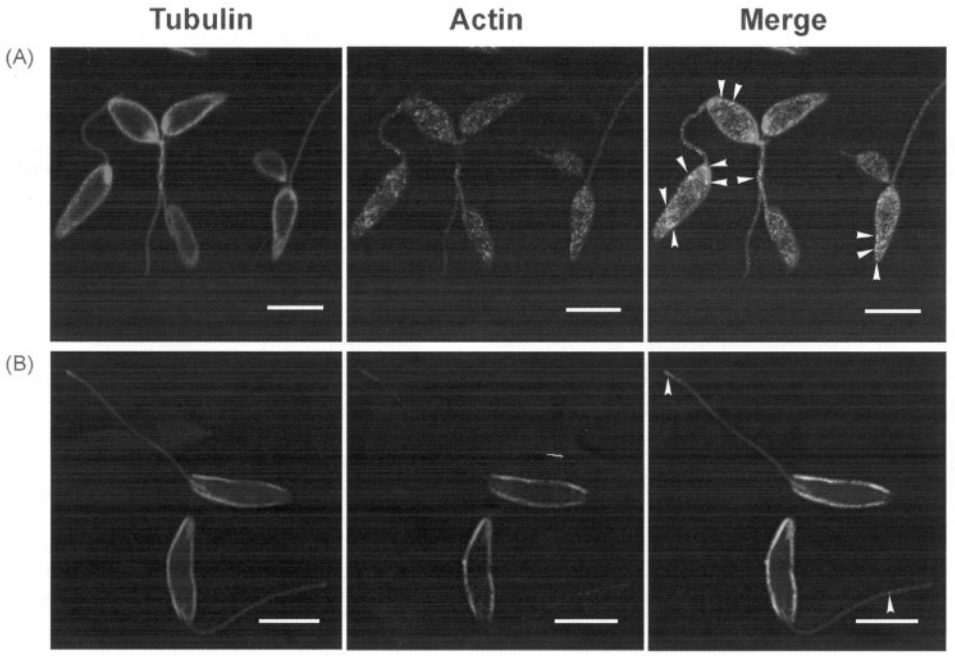

Fig. 9. Immunofluorescence microscopy of *Leishmania* promastigotes (A) and their cytoskeletons prepared by their treatment with 1% Triton X-100 (B) after doubly staining them with anti α,β-tubulin antibodies (red) and anti-*Leishmania* actin antibodies (green). Association of actin with subpellicular microtubules is clearly visible in both the intact cells and cytoskeletons. Bar, 5 μm.

nia actin is a new isoform of actin which may structurally and functionally differ from other eukaryotic actins.

Structural and functional heterogeneity in cytoskeletal proteins is not limited only to actins, but it also exists in other major members of this family of proteins, especially tubulins, which are the key protein components of microtubules. Although the α,β-tubulin isotypes are the main members of this superfamily of proteins, atleast five more members of this family, gamma (γ), delta (δ), epsilon (ξ), zeta (ζ) and eeta (η), have recently been discovered in various eukaryotic organisms [32]. The kinetoplastid parasites, especially *Trypanosoma*, besides containing α,β-tubulins isotypes also contain γ- and ζ-tubulins [33], out of which only γ-tubulin has been functionally characterized [27]. However, so far there is no evidence of the existence of γ-tubulin in *Leishmania* parasites but ζ-tubulin has been identified even in the genome of *L. major* [32]. Such differences between *Leishmania* and *Trypanosoma* may occur also in case of actins, since *Leishmania* cells appear to have only one actin gene, as compared to two or more genes of this protein present in the *Trypanosoma* genome [17]. This may further find support from the various studies reported earlier on ciliates. The ciliate actins, like the *Leishmania* actin, are not stained by phalloidin or recognised by antibodies raised against other eukaryotic actins, and contain diverged amino acids in the DNase I binding loop (aa 40–50) as well as aa 194–200 region [34]. Further, the primary structure and the gene copy number of these actins also vary within this family of organisms [34].

Presence of actin has been reported in the nucleus of several eukaryotic cells, but up till recently, it was mainly ascribed to its thermodynamic equilibrium [4]. However, there is now compelling evidence to suggest that actin in the nucleus exists in form of some yet undefined structures and not in the monomeric form [4,5], which is further corroborated by the present study. As actin oligomerization requires an involvement of specific actin-binding proteins [3,5], we predict that *Leishmania* genome must contain genes that endocde actin-binding proteins required for retaining actin within the nucleus. This finds strong support from the fact that *Leishmania* actin contains two amino acid sequences, viz. aa 171–182 and aa 212–223, which are quite similar to the two nuclear export signals identified earlier in the mammalian and yeast actins [35]. Further, the presence of actin on the nuclear membrane as well as its association, at least in part, with nuclear DNA suggests that this protein could play an important role in chromatin remodelling and RNA transport. This apart, the observed association of actin with kDNA and also with the surface of the kinetoplast indicates that this protein might have been required by the parasite for kDNA network remodelling [36] as well as for the kinetoplast positioning.

Microtubules, rather than the actin microfilaments, regulate the cell morphology at least in trypanosomes [16]. However, no convincing evidence to date is available to completely rule out the role of actin in this process in *Leishmania* parasites [16,17]. Since the *Leishmania* cortical cytoskeleton besides containing α,β-tubulins has also been found in the present study to contain actin, we speculate that actin in *Leishmania* could play a role in maintaining the cell shape by assisting the subpellicular microtubules network in its association with the overlying plasma membrane bilayer. Fur-

ther, the microtubule-actin association may perhaps also be required for the vesicle transport and organelle movement [8,9]. Moreover, the presence of actin in the flagellar pocket [13,14] strongly suggests its role during endocytosis [10,11]. Finally, we speculate that actin in flagella may have a role analogous to that reported earlier in other flagellates [37].

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