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Flagellar localization of a novel isoform of myosin, myosin XXI, in *Leishmania*☆

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ABSTRACT

Leishmania major genome analysis revealed the presence of putative genes corresponding to two myosins, which have been designated to class IB and a novel class, class XXI, specifically present in kinetoplastids. To characterize these myosin homologs in *Leishmania*, we have cloned and over-expressed the full-length myosin XXI gene and variable region of myosin IB gene in bacteria, purified the corresponding proteins, and then used the affinity purified anti-sera to analyze the expression and intracellular distribution of these proteins. Whereas myosin XXI was expressed in both the promastigote and amastigote stages, no expression of myosin IB could be detected in any of the two stages of these parasites. Further, myosin XXI expression was more predominant in the promastigote stage where it was preferentially localized in the proximal region of the flagellum. The observed flagellar localization was not dependent on the myosin head region or actin but was exclusively determined by the myosin tail region, as judged by over-expressing GFP conjugates of full-length myosin XXI, its head domain and its tail domain separately in *Leishmania*. Furthermore, immunofluorescence and immuno-gold electron microscopy analyses revealed that this protein was partly associated with paraflagellar rod proteins but not with tubulins in the flagellar axoneme. Our results, for the first time, report the expression and detailed analysis of cellular localization of a novel class of myosin, myosin XXI in trypanosomatids.

1. Introduction

Leishmania are the causative organisms of several human diseases including kala-azar, and belong to trypanosomatid family of protozoan phyla. These parasites exhibit digenic life cycle, and exist as flagellated promastigotes in the alimentary canal of the sand fly vector and as intracellular aflagellated amastigotes in the human macrophages [1]. The morphological changes observed in different hosts are due to modulation of the flagellum and other cytoskeletal elements [2]. The major cytoskeletal network in these parasites consists of microtubules which envelope the whole cell, except the flagellar pocket region from where the flagellum emerges. The flagellum in trypanosomatids, such as *Leishmania*, is essentially required not only for cell motility but also for morphogenesis and cell signaling [2,3].

Recent studies have shown that besides the microtubules, *Leishmania* also contains actin and actin binding proteins including the actin depolymerizing factor [4–8]. It has further been shown that deletion of the gene that encodes the actin depolymerizing factor results in loss of the assembly and motility of the *Leishmania* flagellum as well as in altered actin distribution [8]. Based on these studies, it has been suggested that actin dynamics is required in the intracellular transport of flagellar proteins from the cytoplasm to the flagellar base and also perhaps in intraflagellar transport [8]. Since myosins are required in actin-dependent intracellular transport by virtue of their actin-based motor activity [9], the present study has been undertaken to analyze the expression and intracellular localization of myosins in *Leishmania*.

Myosins are a diverse group of actin-based motor proteins which are involved in a variety of activities, such as cytokinesis, cell crawling, intracellular trafficking of organelles, vesicles and protein complexes, muscle contraction, etc. [10,11]. These proteins are primarily characterized by their three domains, viz. an N-terminal motor or head domain that binds ATP and filamentous actin, a neck domain which binds light chains or calmodulin, and a C-terminal tail which is highly divergent and serves to anchor and position the motor domain so that it can interact with actin [12]. These proteins are present in all eukaryotic cells including protozoan parasites [13]. Based on the number and structures of domains, myosins have been classified into 24 categories [14]. The Tritryp genome sequence data have revealed that *Leishmania* contains only two isoforms of myosins [15]. While one of the two isoforms is located on chromosome 34 of the *L. major* genome and has been classified to class

Abbreviations: PCR, polymerase chain reaction; GFP, green fluorescence protein; PBS, phosphate buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PFR, paraflagellar rod; DIC, differential interference contrast.

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IB, the other isoform present on chromosome 32 has been recently designated to class XXI [14]. Here we describe our results of expression and intracellular localization of these two isoforms of myosin in *Leishmania donovani*.

2. Materials and methods

2.1. Cells and primers

Leishmania strains were obtained from the National Institute of Immunology, New Delhi, India and maintained in high glucose DMEM, supplemented with 10% foetal bovine serum containing 40 μg/ml gentamycin, at 25 °C. Amastigote stage cells were isolated from the *L. donovani*-infected hamsters and purified by Percoll gradient. The following primers were procured from Sigma, and used in this study.

F1: 5′-CCA TGG GTA TGC CGG AGC GTG TGT CTG TGA-3′
R1: 5′-CTC GAG GCT CAC CTT GAA CAG CAT CTT AAC GGC AG-3′
F2: 5′-GGA TCC ATG GCG TCT GAC TAC AAG CAG CGC GA-3′
R2: 5′-GGA TCC CCG ACT GCC GAG CAC GCT CCG CCA C-3′
F3: 5′-GGA TCC ACC CGT CGT CGC GCG CAG CTG-3′
R3: 5′-GGA TCC AAG TAC CAG CCG CTC CAG C-3′
R4: 5′-GGA TCC ACC CGT CGT CGC GCG CAG CTG-3′
F5: 5′-CCA TGG CAT GTT TCA CTC TGC AGA AGC GC-3′
R5: 5′-GTC GAC GCT CGT CAG CTT CAC CTC G-3′

2.2. Cloning and expression of L. donovani myosin isoform located on chromosome 32 (myo21) and also of the variable region of the myosin located on chromosome 34 (myo1Bv)

Gene specific primers were designed on the basis of *L. major* myosin gene present on chromosome 32. The putative myosin gene of 3.15 kb was amplified by polymerase chain reaction (PCR) using high fidelity platinum Pfx polymerase (Invitrogen, USA), *L. donovani* genomic DNA as the template, and primers F1 and R1. Blunt-end PCR product generated was 'A' tailed using Taq DNA polymerase for T/A ligation in the pGEMT-EASY vector and sequenced by dideoxy chain termination method. The gene sequence was submitted to NCBI gene databank (accession No. FJ028724). Putative myosin gene was subsequently cloned in pET21d vector at NcoI and XhoI sites. A portion of myosin IB gene (myo1Bv) from 3040 to 3639 bases was amplified by using primers F5 and R5 by Taq DNA polymerase and was cloned in pTZ57R vector by T/A ligation. Both the fragments were subsequently cloned in pET21d vector at NcoI and XhoI sites and over-expressed in *Escherichia coli* strain BL21 (DE3) by inducing grown cultures (0.4 OD_{600}) with isopropyl β-D-thiogalactopyranoside (0.5 mM) for 5 h at 37 °C.

2.3. Antibodies

The recombinant myo21 and myo1Bv overexpressed in *E. coli* were present as an aggregate in the inclusion bodies. Pure protein from the aggregates was isolated by preparatory SDS-polyacrylamide gel electrophoresis (PAGE). The purified recombinant myo21 was injected into rabbits and mice, and myo-1BV was injected into rats to raise the polyclonal antiserum. Anti-*Leishmania* myo21 and anti-*Leishmania* myo1Bv antibodies were affinity purified using recombinant proteins. Antibodies against α and β-tubulins (monoclonals) and anti-GFP antibodies were procured from Sigma and Molecular probes, USA, respectively. Anti-paraflagellar rod proteins antibodies were a kind gift from Dr. McMahon Pratt. Anti-*Leishmania* actin antibodies were prepared as described earlier [4].

2.4. Expression of GFP fusion proteins of myo21 as well as of its head and tail domains in Leishmania

The myo21 gene was PCR amplified using primers F2 and R2 from the *L. donovani* genome and cloned in frame to the N-terminus of the GFP gene in pXG-'GFP+ vector at BamHI site [16]. The N-terminus of the gene which includes the conserved motor domain region (67–747 amino acids) from 1 to 2250 bases (Head) and the C-terminus portion (tail) of the gene from 2250 to 3150 bases were cloned as fusion product, with GFP in pXG-'GFP vector after DNA amplification using primers F2, R3, F3 and R4. GFP was attached at the C-terminus of the head (myo21H) domain of the gene, whereas the tail region (myo21T) carried GFP at its N-terminus. Cloned plasmid constructs were electroporated in log phase *L. donovani* promastigotes and the selectants were obtained by gradually increasing G418 concentration from 10 μg to 50 μg per ml in the culture.

2.5. Western blotting and NP-40 extraction

L. donovani promastigote and amastigote stage cells were used to examine the expression of myo21 in *Leishmania*. The promastigotes (5×10^7 cells) were washed with PIPES buffer (100 mM PIPES, 2 mM $MgCl_2$ and 1 mM EGTA). The washed cells were incubated in PIPES buffer containing 1% NP-40 at 4 °C for 5 min and centrifuged at 13,000 × *g* for 10 min at 4 °C. Supernatant was collected and the pellet was washed once in the above buffer. Whole cell lysates, soluble, and cytoskeletal protein fractions were analyzed by SDS-polyacrylamide gel (8%) electrophoresis. The gels were blotted on to the PVDF membrane and probed with anti-myo21 or anti-GFP antibodies. The protein bands were detected using Millipore chemi-luminiscent reagent.

2.6. Immunofluorescence

Cells were washed in phosphate-buffered saline (PBS), and allowed to adhere on poly-L-lysine coated glass cover slips. Adhered cells were fixed using paraformaldehyde solution (4%) for 30 min and washed with PBS containing 0.5% glycine (w/v). The washed cells were permeabilized using 0.5% (v/v) Triton X-100 and after blocking with 0.5% bovine serum albumin (BSA) in PBS, the cells were labeled using primary antibodies. The labeled cells were washed in blocking buffer to remove non-specifically bound antibodies and again labeled with secondary antibodies conjugated to Alexa 488 or Cy3. For co-labeling of myo21 and paraflagellar rod (PFR) proteins, the cells were fixed in chilled methanol and permeabilized with acetone and air-dried. The methanol free cells were hydrated in PBS for 15 min and subsequently labeled as above.

2.7. Immuno-gold electron microscopy

L. donovani promastigotes were fixed with paraformaldehyde (4%, w/v) and glutaraldehyde (0.1%, w/v) in PBS. This was followed by two more changes of the fixing reagents and subsequent incubation for 4 h. The excess fixatives were quenched with 0.5% glycine in PBS (pH 7.4) at 4 °C for 10 min and the fixed cells were 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-myo21 antibodies 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