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Supplementary

Supplementary Fig.1.

Generation and Characterization of Rab7mutants from L. donovani

To investigate the role of LdRab7 in Hb trafficking in *Leishmania*, three mutants *viz*, GTP-locked (Q66L), GDP-locked (T21N) and isoprenylation deficient (ΔC) were generated by PCR mediated site directed mutagenesis approach using appropriate mutant primers and LdRab7:WT as a template. LdRab7:Q66L and LdRab7:T21N were generated by using appropriate mutant primer where CAG codon at position 66 was changed to CTG and ACG codon at position 21 was changed to AAT, respectively.

Methods:

Generation of Rab mutants:

In the first round of PCR, a megaprimer was amplified using the appropriate forward mutant primer (5'-ACGGCCGGGCTGGAGCGGTTC-3') or (5'-GTCGGCAAGAATTCG CTCATG-3') respectively, and the reverse WT primer. The amplified respective megaprimer was gel purified using QiaGen Gel purification kit. Subsequently, a second PCR was performed with WT forward primer and the respective megaprimer as reverse primer using LdRab7:WT as template to amplify the full-length LdRab7:Q66L and LdRab7:T21N mutants. The PCR was initially performed for 5 cycles of denaturation at 94 °C for 1 min followed by extension at 72 °C for 1 min to allow synthesis of the megaprimer strand. Subsequently, 25 pmoles/μl of the Rab7 forward primer was added while at 72 °C, and PCR was continued for 25 cycles using the same conditions as described in previous section. To generate LdRab7:ΔC mutant, PCR was carried out under same conditions using WT forward primer and mutant reverse primer (5'-GAATTCTTAGGCGGAGGAGGTGG-3'), in which the nucleotides corresponding to the last two C-terminal cysteine residues were deleted. PCR products were sequenced to confirm the respective mutations and sub-cloned into the *Bam*HI/*Eco*RI sites of pGEX4T-2 to express as GST fusion proteins.

GTP overlay assay

GTP binding ability of purified LdRab7 and its mutants was determined by GTP overlay assay (Singh et al., 2003). Briefly, indicated amounts respective proteins blotted onto nitrocellulose membranes were incubated with 1μCi/ml of [α-^{32}P]GTP (3000 Ci/mM, NEN) in GTP binding buffer (50 mM phosphate buffer, pH 7.5 containing 5 mM $MgCl_2$, 1 mM EGTA and 0.3% Tween-20) for 3 h at RT, washed and visualized by autoradiography.

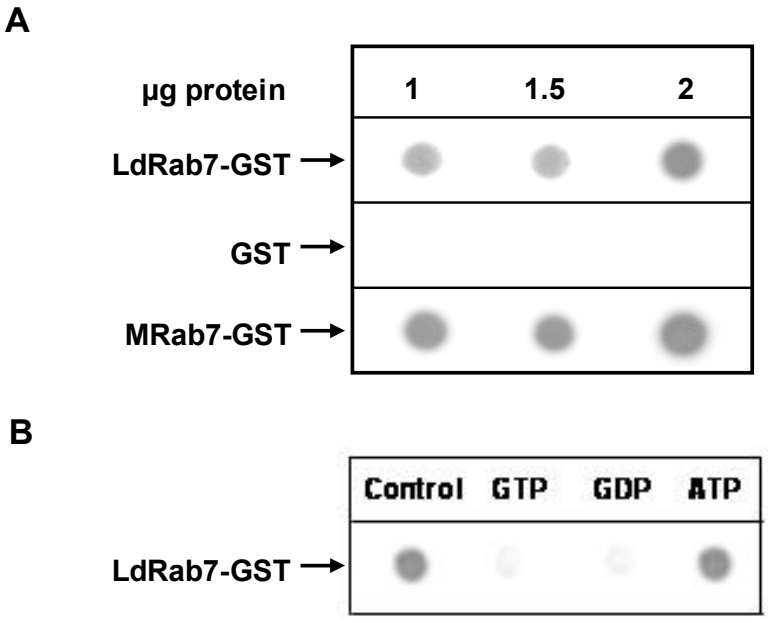
GTPase assay

The GTPase activity of LdRab7 and its mutants was determined using the procedure described (Singh et al., 2003). Briefly, 2 pmol of indicated proteins were immobilized and incubated with buffer A (20mM Tris-HCl, pH7.8, 100mM NaCl, 5mM $MgCl_2$, 1mM Na-phosphate and 10mM 2-mercaptoethanol) for 20 min at 25ºC and bound nucleotides were eluted with 1M guanidine-HCl. Immobilized proteins were then

incubated with 2 pmol [α-^{32}P]GTP (800 Ci/mmol, Perkin Elmer Life Sciences, MA) in 20µl buffer A for 10 min at 0ºC followed by 1h at 23ºC. Subsequently, samples were washed and incubated in 8µl of buffer B (0.2%SDS, 2mM EDTA, 10mM GDP, 10mM GTP, pH7.5) and heated for 2 min at 70ºC. An aliquot was analyzed to determine the degree of hydrolysis using thin layer chromatography and visualized by autoradiography.

Results:

GTP binding activity of GST-LdRab7 was determined using [α-^{32}P]-GTP. The results presented in the Fig.1A showed that unlike free GST, GST-LdRab7 binds significant amount of [α-^{32}P]-GTP, almost comparable to that of mouse Rab7. Moreover, the binding of [α-^{32}P]-GTP with LdRab7 was specific as this binding was competed out by unlabeled GTP and GDP but not by ATP (Fig.1B). In order to further characterize the role of LdRab7 in Hb trafficking in *Leishmania*, three mutants were generated by site-directed mutagenesis based on the previous knowledge of analogous mutations in mammalian Rabs (Press *et al.,* 1998). The LdRab7:T21N mutant was generated by substituting asparagine for threonine in the GKT/S region, whereas leucine was substituted for glutamine in the WDTAGQE region in LdRab7:Q66L mutant. LdRab7:ΔC mutant was generated by deletion of last cysteine residues from the C-terminus. Subsequently, GTP binding ability and GTPase activity of these mutants were checked. Our results showed that LdRab7:Q66L and LdRab7:ΔC mutants bind [α-^{32}P]-GTP comparable to LdRab7:WT protein whereas LdRab7:T21N fails to bind GTP (Fig.1C). Moreover, analysis of GTPase activity of these mutants revealed that LdRab7:WT hydrolyze GTP to GDP like any other RabGTPases, whereas, GTP hydrolysis is blocked in LdRab7:Q66L mutant (Fig.1D). Moreover, LdRab7:Q66L mutant protein binds GTP similar to the wild type LdRab7, however, it is unable to hydrolyze GTP. In contrast, LdRab7:T21N exhibits marked reduction in GTP binding relative to the wild-type protein.



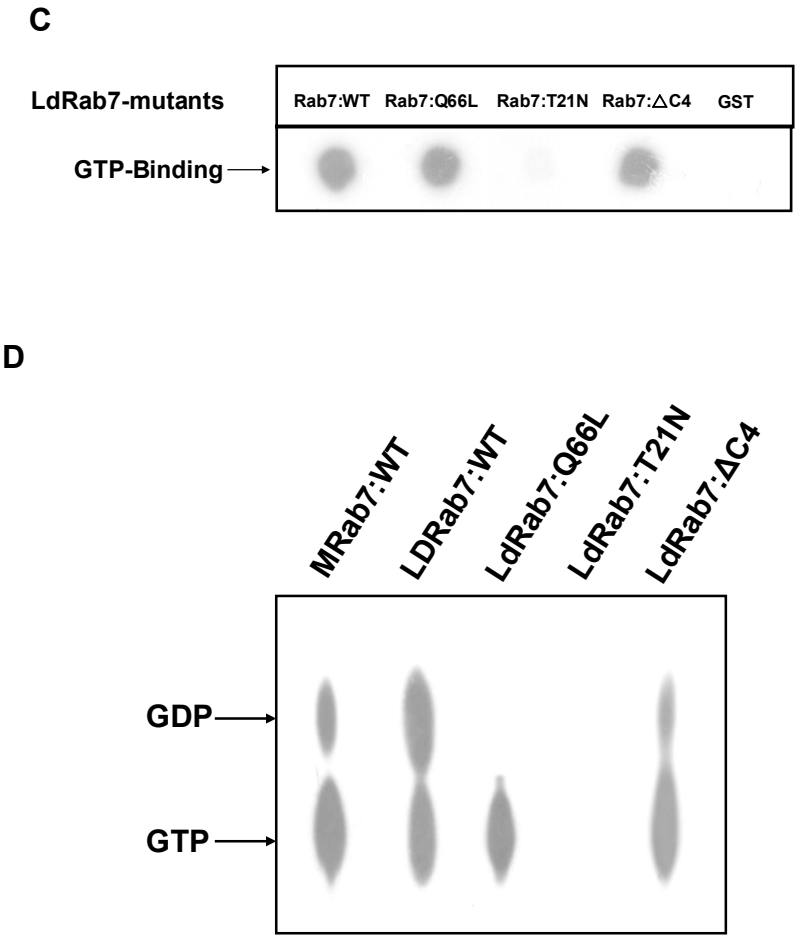


Fig. 1. Characterization of Rab7 from *L. donovani*: (**A**) GTP binding of indicated concentrations of LdRab7 was detected using [α-^{32}P]GTP overlay assay. Free GST and mammalian Rab7 were used as control. (**B**) Specificity of [α-^{32}P]GTP binding with LdRab7:WT was measured in the absence (control) or presence of 1mM of indicated nucleotides. (**C**) GTP binding activity of LdRab7 and its mutants were determined as stated in Materials and Methods. Free GST was used as control. (**D**) GTPase activity of LdRab7 and its mutants were determined as stated in Materials and Methods. Mammalian Rab7:WT protein was used as control. Results are representative of three independent preparations.

Reference:

1. Press B, Feng Y, Hoflack B, Wandinger-Ness A (1998) *J. Cell Biol.* 140: 1075-1089.
2. Singh SB, Tandon R, Krishnamurthy G, Vikram R, Sharma N, Basu SK, Mukhopadhyay A (2003) *EMBO J.* 22: 5712-5722.

Supplementary Fig. 2.

Overexpression of Rab7 and its mutants in Leishmania

The overexpression of different forms of LdRab7 as fusion proteins in *Leishmania* in comparison to untransfected control cells were determined by Western blot analysis of the cell lysates using specific antibodies against LdRab7 and GFP.

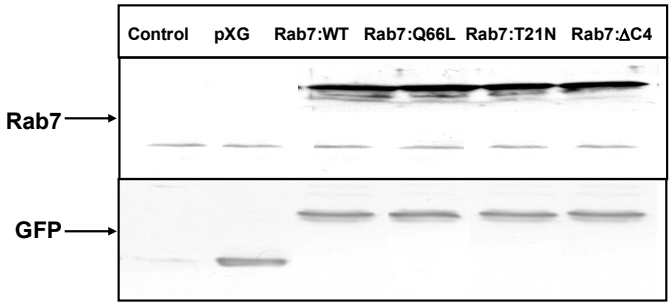


Fig.2. LdRab7 and its mutants were overexpressed in *Leishmania* promastigotes as described in Materials and Methods. Overexpression of LdRab7 and its mutants as a GFP fusion proteins were determined by Western blot analysis using indicated antibodies from the respective cell lysates.

Results:

The lysates prepared form cells transfected with different forms of LdRab7 showed about 5-6 folds higher expression of the respective Rab7 as GFP fusion protein in comparison to control cells (Fig. 2).

Supplementary Fig. 3.

Detection and localization of endogenous Rab7 in Leishmania

To determine the localization of LdRab7 in *Leishmania*, we used indirect immunofluorescence assay using anti-LdRab7 antibodies as described in materials and methods.

Methods:

Leishmania promastigotes (1×10^7 cells/ml) were harvested from the exponential growth condition by centrifugation for 10 min at 800g. The cell pellet was washed and resuspended in Voorheis's modified-PBS (vPBS) (136.9mM NaCl, 3mM KCl, 16mM Na_2HPO_4, 3mM KH_2PO_4, 45.9mM sucrose, 10mM glucose pH 7.6). The cells were then fixed with 3% paraformaldehyde for 1 h on ice. Subsequently, the cells were washed twice and a thin smear of cells was prepared on poly-lysine coated slides. Finally, parasites were permeabilized in PBS containing 0.1% triton X-100 for 10 min. After washing, cells were incubated with 20% FCS for 1 h at RT to block non-specific binding

sites. Subsequently, cells were incubated with appropriate dilutions (1:200) of primary anti-LdRab7 antibody for 1 h at RT. Cells were washed thrice and probed with Alexa Fluor 488 labeled goat anti-mouse secondary antibodies in the same buffer for 1 h to detect the signal. Finally, the slides were washed and incubated with 5µg/ml of propidium iodide (PI) for 20 min in order to visualize the nucleus and kinetoplast. The slides were then mounted with an antifade reagent and viewed with a Zeiss LSM 510 META confocal microscope.

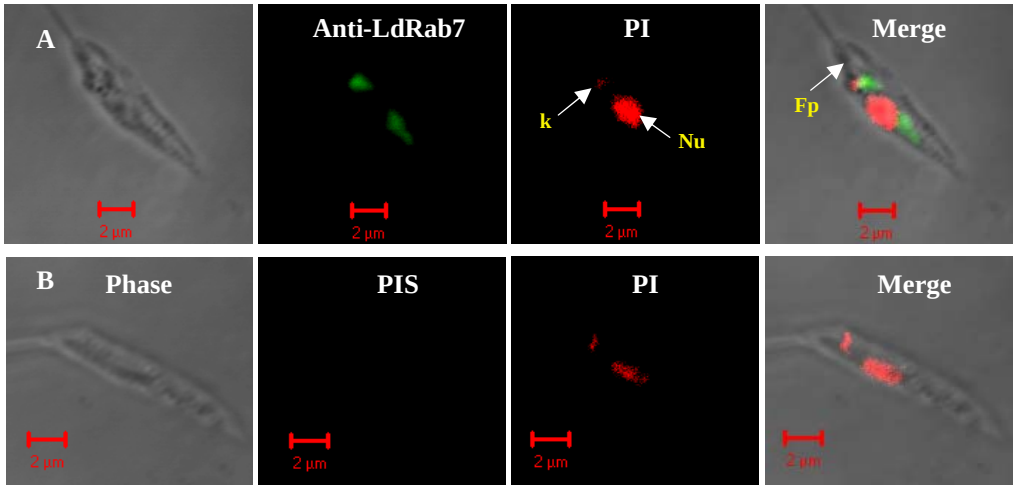


Fig. 3. Immunolocalization of endogenous Rab7 in *Leishmania* promastigotes. Cells were permeabilized and endogenous protein was visualized using anti-LdRab7 antibodies (A) or pre-immune serum (B). The cells were co-stained for nucleus (Nu) and kinetoplast (k) by propidium iodide.

Results:

The results presented in Fig. 3 show that endogenous Rab7 is mainly localized in the perinuclear compartments with additional labeling in the early endocytic compartment near kinetoplast as observed with overexpression of LdRab7 as GFP fusion protein. Thus, addition of GFP tag with Rab7 does not alter the localization of this protein in *Leishmania* and thereby these cells are used to determine the role of Rab7 in hemoglobin trafficking in *Leishmania.*