

# Rab5 Isoforms Specifically Regulate Different Modes of Endocytosis in *Leishmania*\*

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Differential functions of Rab5 isoforms in endocytosis are not well characterized. Here, we cloned, expressed, and characterized Rab5a and Rab5b from *Leishmania* and found that both of them are localized in the early endosome. To understand the role of LdRab5 isoforms in different modes of endocytosis in *Leishmania*, we generated transgenic parasites overexpressing LdRab5a, LdRab5b, or their dominant-positive (LdRab5a:Q93L and LdRab5b:Q80L) or dominant-negative mutants (LdRab5a:N146I and LdRab5b:N133I). Using LdRab5a or its mutants overexpressing parasites, we found that LdRab5a specifically regulates the fluid-phase endocytosis of horseradish peroxidase and also specifically induced the transport of dextran-Texas Red to the lysosomes. In contrast, cells overexpressing LdRab5b or its mutants showed that LdRab5b explicitly controls receptor-mediated endocytosis of hemoglobin, and overexpression of LdRab5b:WT enhanced the transport of internalized Hb to the lysosomes in comparison with control cells. To unequivocally demonstrate the role of Rab5 isoforms in endocytosis in *Leishmania*, we tried to generate null-mutants of LdRab5a and LdRab5b parasites, but both were lethal indicating their essential functions in parasites. Therefore, we used heterozygous LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells. LdRab5a<sup>+/-</sup> *Leishmania* showed 50% inhibition of HRP uptake, but hemoglobin endocytosis was uninterrupted. In contrast, about 50% inhibition of Hb endocytosis was observed in LdRab5b<sup>+/-</sup> cells without any significant effect on HRP uptake. Finally, we tried to identify putative LdRab5a and LdRab5b effectors. We found that LdRab5b interacts with clathrin heavy chain and hemoglobin receptor. However, LdRab5a failed to interact with the clathrin heavy chain, and interaction with hemoglobin receptor was significantly less. Thus, our results showed that LdRab5a and LdRab5b differentially regulate fluid phase and receptor-mediated endocytosis in *Leishmania*.

Endocytosis is a fundamental process and is required for the uptake of various essential molecules for the maintenance of

cells (1, 2). After initial binding with cell surface receptors, endocytic cargos are internalized into common early endosome (EE)<sup>3</sup> where they are sorted and targeted to various intracellular destinations (3); however, the mechanism of sorting is not well characterized (4). Moreover, the route of internalization of different ligands also controls the fate of endocytic cargos (5). Therefore, it could be possible that ligand-receptor interaction might activate or modulate the function of certain endocytic Rab-GTPases to dictate its endocytic route as Rab-GTPases are master regulators of intracellular trafficking (6).

Among the large number of Rab proteins, Rab5 is specifically localized to EE and regulates endocytosis (7, 8). Incidentally, three Rab5 isoforms, namely Rab5a, Rab5b, and Rab5c, are present in mammalian cells (9). Initial studies have shown that overexpression of individual Rab5 isoforms stimulates transferrin endocytosis (9), and simultaneous knockdown of all three Rab5 isoforms are required to inhibit insulin-dependent PKB/Akt activation suggesting their functional redundancy (10). However, recent studies have shown that Rab5 isoforms might have different roles in different cell types. For instance, Rab5a has been shown to specifically regulate epidermal growth factor receptor endocytosis in HeLa cells (11, 12), although in osteosarcoma cells, Rab5a interacts with Beclin-1 to regulate autophagosome formation (13). In contrast, Rab5a is also found to regulate trafficking of lysosomal enzymes (14) and induces the transport of intracellular pathogens to lysosomes in macrophages (15). Rab5b is also found to regulate the biogenesis of lysosomes in rat kidney cells (16). However, Rab5c has been shown to enhance  $\beta$ -integrin recycling in EGF-induced cancer cell invasion (17) possibly by increasing cell motility by Rac1 activation (18). Furthermore, expression of Rab5 isoforms can be differentially regulated by diverse cytokines (19, 20). As all isoforms of Rab5 are localized on EE, it is tempting to speculate that Rab5 isoforms might play differential roles in endocytosis of various cargos. However, the role of Rab5 isoforms in regulating various types of endocytosis in a particular cell type has not yet been addressed.

It has been shown that large numbers of putative Rab and SNARE proteins are also present in trypanosomatid parasites indicating the presence of highly conserved protein trafficking pathways in these parasites (21, 22). Previously, we have shown that *Leishmania* endocytosed hemoglobin (Hb) via receptor-

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<sup>3</sup> The abbreviations used are: EE, early endosome; RME, receptor-mediated endocytosis; FPE, fluid-phase endocytosis; LdChC, clathrin heavy chain of *Leishmania*; HbR, Hb receptor; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein.

mediated endocytosis (RME) (23) through a specific receptor located in the clathrin-coated flagellar pocket (24, 25). Subsequently, bound hemoglobin is rapidly internalized into discrete Rab5-positive EE (26) and finally targeted to the lysosomal compartment in a Rab7-dependent way (27). However, which Rab5 isoform regulates the Hb endocytosis in *Leishmania* was not characterized. We have also found that the Rab1-mediated conventional secretory pathway is conserved in *Leishmania* (28). These results demonstrate that several components of intracellular trafficking machinery are well preserved in *Leishmania*; thereby, this parasite provides a unique opportunity to determine the intricacies of membrane trafficking and sorting phenomena in an organism. Therefore, we have analyzed the role of Rab5 isoforms in RME and fluid-phase endocytosis (FPE) in *Leishmania* promastigotes using Hb and HRP as endocytic probes, respectively, and have shown that Rab5 isoforms differentially regulate these two endocytic pathways.

### Experimental Procedures

**Materials**—Unless otherwise stated, all reagents were obtained from Sigma. Platinum High Fidelity *Taq* polymerase and restriction enzymes were purchased from Invitrogen and Promega Life Science (Madison, WI), respectively. Glutathione-Sepharose 4B beads, protein markers (RPN756 and RPN800), and ECL reagents were obtained from Amersham Biosciences, UK. Dextran-Texas Red (10,000 Da), Alexa Fluor-594 succinimidyl ester, FM4-64, LysoTracker Green, and anti-GFP antibody were obtained from Molecular Probes (Eugene, OR). Geneticin and hygromycin were procured from Gibco BRL (Gaithersburg, MD). The *Leishmania* expression vectors, pXG-GFP<sup>2+</sup> and pXG were kindly provided by Dr. S. M. Beverley (Washington University, St. Louis, MO), and pGL345-Hyg vector was gift from Dr. Jeremy Mottram (University of Glasgow, Glasgow, UK). pNUS-mRFP-nD was a kind gift from Dr. Jean-Paul di Rago (Institut de Biochimie et Génétique Cellulaires, Bordeaux, France). [ $\alpha$ -<sup>32</sup>P]GTP (800 Ci/mmol) was procured from PerkinElmer Life Sciences. All other reagents used were of analytical grade.

**Cells**—*Leishmania donovani* (UR6) and *L. donovani* (Bob strain) promastigotes were obtained from the Indian Institute of Chemical Biology, Kolkata, and Jawaharlal Nehru University, Delhi, India, respectively. Cells were routinely maintained on blood agar slants containing glucose, peptone, sodium chloride, beef heart extract, rabbit blood, and gentamycin as described previously (27). For experiments, cells were cultured in medium M199, pH 7.4, supplemented with 10% FCS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin at 23 °C, and log-phase cells were harvested in phosphate-buffered (10 mM, pH 7.2) saline (0.15 M).

**Cloning and Expression of *rab5a* from *L. donovani* (*Ldrab5a*)**—To clone Rab5a from *Leishmania*, a putative Rab5a sequence was identified from a *Leishmania major* genome with substantial homology with *Trypanosoma brucei* Rab5a by BLAST search. Accordingly, appropriate forward (5'-GCGGATCCATGAATACCCACCCACCTCAGC-3') and reverse (5'-GCCGAATTCTCAGCAGCGTAGACTGGGAG-3') primers, including start or stop codons, were designed against the putative LdRab5a gene sequence from *L. major*. These primers

were used to amplify the *Ldrab5a* gene sequence from *L. donovani* cDNA by RT-PCR. Briefly, PCR was performed in a PerkinElmer Life Sciences thermocycler for 30 cycles (denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 68 °C for 1 min) using High Fidelity *Taq*. The PCR product was cloned into pGEM-T Easy vector (Promega Life Science) as per the manufacturer's protocol and sequenced using M13 universal primers. After confirming the sequence, the LdRab5a gene product was further subcloned into BamHI/EcoRI sites of the pGEX-4T-2 (Amersham Bioscience) expression vector and transformed into the XL-1 Blue strain of *Escherichia coli*.

**Generation of *LdRab5* Mutants**—The dominant-positive (Gln to Leu) and dominant-negative (Asn to Ile) mutants of LdRab5a and LdRab5b were generated by the PCR-mediated site-directed mutagenesis approach as described previously (27). Briefly, a mutant primer was designed in such a way that the Gln residue at position 93 changed to an Leu residue in LdRab5a (5'-GAAGCGTTCCAGCCCCGCG-3'). PCR (30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 68 °C for 30 s) was carried out using this mutant primer as reverse primer and Rab5a:WT primer as forward primer to amplify a megaprimer (290 bp). The amplified megaprimer was gel-purified, and a second PCR was carried out to amplify the full-length LdRab5a:Q93L mutant (~708 bp) using the megaprimer as a forward primer and a gene-specific reverse primer. Similarly, LdRab5a:N146I mutant was generated using mutated primer (5'-GTGCTCGTCGGAATCAAG-AAGGACCTGGGG-3') as forward primer and Rab5a:WT primer as reverse primer in the first round of PCR. Additionally, LdRab5b:Q80L and LdRab5b:N133I were made by similar methods using (5'-CTTAAAGCGCTCCAGCCCTGCCGTGTC-3') and (5'-CTGGTAGGCATCAAGAAGGACTTG-3') as mutated primer, respectively.

The full-length PCR products were subsequently cloned into pGEM-T Easy and sequenced to confirm the respective mutations. Subsequently, mutants were subcloned into pGEX-4T-2 vector and transformed into *E. coli*. Cells were grown in LB and induced with 0.5 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside for 3 h at 37 °C for expression of respective GST fusion proteins using reduced glutathione beads by standard procedure.

**Generation of Antibodies against *LdRab5a* and *LdRab5b***—LdRab5a and LdRab5b were subcloned into pET28a and expressed as His<sub>6</sub> tag proteins. 10  $\mu$ g of the respective proteins were immunized in BALB/c mice to raise antibodies by a standard method as described previously (26). The specificity of the antibodies against the respective proteins was determined by Western blot analysis using the respective purified proteins.

**Preparation of Early Endosomes from *L. donovani***—Early endosomes containing biotinylated-Hb or avidin-HRP were purified from *Leishmania* as described previously (26). Briefly, cells were incubated with biotinylated-Hb (2 mg/ml) or avidin-HRP (2 mg/ml) in internalization medium (MEM containing 10 mM HEPES and 5 mM glucose, pH 7.4) for 5 min at 23 °C to label the early endosomal compartment. Cells were washed three times with cold homogenization buffer (HB: 20 mM HEPES, 250 mM sucrose, and 2 mM EGTA, pH 7.2, containing protease inhibitors) and disrupted by release of N<sub>2</sub> from a pre-cooled nitrogen cavitation bomb. The unbroken cells, nuclei, and

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other cell debris were removed by low speed centrifugation at  $500 \times g$  for 10 min at 4 °C. The post-nuclear supernatant (0.5 ml) was loaded onto a discontinuous sucrose density gradient formed by layering 0.35 ml of 54%, 1.45 ml of 40%, and 1.45 ml of 30% sucrose in HB. After centrifugation in an MLS 50 rotor (Beckman TL100) at  $100,000 \times g$  for 1 h at 4 °C, 50- $\mu$ l fractions were collected from the top of the gradient as described previously. Early endosomal fractions containing avidin-HRP or biotinylated-Hb were analyzed by Western blot analysis using anti-Rab5a or anti-Rab5b antibodies, respectively.

**GTP Binding Assay**—GTP binding activity of purified LdRab5a:WT, LdRab5b:WT, and their mutants was detected by a GTP binding assay (26). Briefly, the indicated proteins (2  $\mu$ g) were blotted onto nitrocellulose membrane, and the membrane was incubated with 1  $\mu$ Ci/ml [ $\alpha$ - $^{32}$ P]GTP in 50 mM phosphate buffer, pH 7.5, containing 5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.3% Tween 20 for 3 h at 24 °C. Finally, the membranes were extensively washed to remove unbound radioactivity and visualized by autoradiography.

**GTPase Assay**—The GTPase activity of the indicated proteins was determined as described previously (26). Briefly, 5  $\mu$ g of respective protein was immobilized on glutathione beads, and beads were incubated with buffer A (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM  $\beta$ -mercaptoethanol) for 20 min at 25 °C. The bound nucleotide was eluted with 1 M guanidine HCl. Nucleotide-free immobilized protein was then loaded with 2 pmol of [ $\alpha$ - $^{32}$ P]GTP (800 Ci/mmol) in 20  $\mu$ l of buffer A for 10 min at 0 °C. Subsequently, beads were washed and incubated for 1 h at 23 °C to allow the hydrolysis of bound GTP. The beads were washed, incubated in 8  $\mu$ l of buffer B (0.2% SDS, 2 mM EDTA, 10 mM GDP, 10 mM GTP, pH 7.5), and heated at 70 °C for 2 min to elute the nucleotide from the protein. An aliquot was analyzed using thin layer chromatography and visualized by autoradiography.

**Overexpression of LdRab5 Isoforms and Their Mutants in *L. donovani* Promastigotes**—To overexpress LdRab5a, LdRab5b or its mutants in *Leishmania* promastigotes, the full-length *Ldrab5a*, *Ldrab5b*, or its mutant gene was subcloned into pXG vector and pXG-GFP<sup>+</sup>2 vector to express the proteins without tag or with an N-terminal GFP tag, respectively (29). For subcloning, the respective construct was first linearized by digestion with BamHI and subsequently partially digested with EcoRI followed by ligation with BamHI-EcoRI-digested LdRab5 or its mutants. In addition, *Ldrab5a* was also subcloned into BglII/XhoI sites of pNUS-mRFP-nD vector to express as RFP fusion protein. *Leishmania* promastigotes were then transfected with respective constructs, using standard protocol (27). Briefly, *Leishmania* cells were suspended at a density of  $1.0 \times 10^8$ /ml in HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH<sub>2</sub>PO<sub>4</sub>, and 6 mM glucose, pH 7.4). Cells (0.4 ml) were transferred to pre-cooled electroporation cuvette containing appropriate construct of chilled DNA (40  $\mu$ g), followed by electroporation using a GenePulser (Bio-Rad) to facilitate DNA uptake by cells. Cells were allowed to recover on ice for 10 min and transferred into drug-free medium for 30 h at 23 °C. Subsequently, stable clones were selected in the presence of G418 antibiotic (50  $\mu$ g/ml). Overexpression of the respective

protein was confirmed by Western blot analysis and by confocal microscopy.

**Subcellular Localization of LdRab5a and LdRab5b in *L. donovani***—To characterize the subcellular localization of LdRab5a and LdRab5b, cells overexpressing GFP-LdRab5a or GFP-LdRab5b proteins were labeled with various compartment-specific markers. The flagellar pocket was labeled by incubating the promastigotes with FM4-64 (30  $\mu$ M) for 5 min at 4 °C. Similarly, 5 min of internalization of Alexa Fluor-594-labeled hemoglobin (Alexa 594-Hb) and 10 min internalization of dextran-Texas Red at 23 °C were used to mark the EE. Lysosomes were stained with 100 nM LysoTracker Red at 4 °C for 5 min. Overnight serum-starved *Leishmania* were incubated with 5  $\mu$ M BODIPY-TR ceramide bound to defatted bovine serum albumin for 1 h at 23 °C to label the Golgi complex. Finally, cells were fixed with 4% paraformaldehyde on ice for 20 min, washed with vPBS (136.9 mM NaCl, 3 mM KCl, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM KH<sub>2</sub>PO<sub>4</sub>, 45.9 mM sucrose, and 10 mM glucose, pH 7.4), and visualized under a Zeiss LSM 510 META confocal microscope.

**Fluid Phase Uptake of HRP**—HRP was used to measure the fluid-phase endocytosis in *Leishmania* promastigotes. Log-phase *Leishmania* promastigotes were harvested, washed, and resuspended in vPBS containing 1 mg/ml BSA at a cell density of  $8 \times 10^7$  cells/ml. Subsequently,  $2 \times 10^7$  cells were incubated with different concentrations of HRP at 23 °C for 60 min. The cells were then washed four times with chilled vPBS containing 10 mg/ml BSA followed by two washes with chilled vPBS to remove uninternalized HRP. Finally, cells were transferred into fresh tubes and lysed by the addition of 50  $\mu$ l of solubilization buffer (SB: PBS containing 0.5% Triton X-100, 2% methylbenzethonium chloride). An aliquot (10  $\mu$ l) of the cell lysate was used to measure the uptake of HRP by respective cells using a standard assay procedure (30). Results were expressed as nanograms of HRP per mg of cell protein.

**Kinetics of Dextran-Texas Red Trafficking in *L. donovani***—To measure the kinetics of fluid phase endocytosis, fluorescently labeled dextran-Texas Red was used. Respective *Leishmania* promastigotes ( $2 \times 10^7$  cells/ml) were washed twice with vPBS and resuspended in 250  $\mu$ l of vPBS containing 150  $\mu$ g/ml dextran-Texas Red for 10 min. The cell were washed twice with vPBS and incubated for the indicated periods of time at 23 °C. Finally, cells were washed and incubated with 100 nM LysoTracker Green for 5 min on ice. Cells were fixed with 4% paraformaldehyde on ice for 20 min, washed with vPBS, and visualized under a Zeiss LSM 510 META confocal microscope.

**Uptake and Degradation of  $^{125}$ I-Hemoglobin by *L. donovani* Promastigotes**—To determine the role of LdRab5a and LdRab5b in Hb endocytosis, uptake and degradation of  $^{125}$ I-Hb by *Leishmania* promastigotes was measured in cells overexpressing either GFP-LdRab5a or GFP-LdRab5b along with vector-transfected control cells (24). Briefly, cells ( $2 \times 10^7$  cells/ml) were resuspended in vPBS containing 1 mg/ml BSA and incubated with 6  $\mu$ g/ml  $^{125}$ I-Hb for different time intervals at 23 °C. At respective time intervals, cells were washed to remove the unbound radioactivity, and the cell pellet was dissolved in 0.1 N NaOH. An aliquot was used to determine the cell-associated radioactivity. To determine the degradation of internalized Hb

by *Leishmania*, aliquots of the supernatant from the respective medium were processed for the determination of trichloroacetic acid-soluble non-iodide radioactivity after extraction with chloroform. Results were expressed as nanograms of Hb/mg of cell protein.

Hb endocytosis in LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells was also measured using biotinylated Hb. Briefly, the respective cells were incubated with 6 μg/ml biotinylated Hb for 2 h at 23 °C and washed. Cells were solubilized in SB, and Hb was immunoprecipitated from the cell lysate using anti-Hb antibody. The amount of internalized biotinylated Hb was quantitated using avidin-HRP as described previously (25). Results were expressed as percentage uptake of Hb in indicated cell types considering 100% in the control cells.

**Kinetics of Intracellular Trafficking of Hb in *L. donovani***—Kinetics of intracellular trafficking of Hb in *Leishmania* was determined as described previously (27). Briefly, respective *Leishmania* cells (10<sup>7</sup> cells/ml) were washed twice and resuspended in 250 μl of ice-cold vPBS containing Alexa Fluor-594-labeled Hb (120 μg/ml) and incubated at 23 °C for 5 min to label the early endosomal compartment. Cells were washed three times with cold vPBS to remove unbound Alexa-Hb and resuspended in pre-warmed (23 °C) vPBS for the indicated periods of time. At respective times, cells were transferred onto ice and washed twice with chilled vPBS. The lysosomes of the cells were stained with 100 nM LysoTracker Green for 5 min on ice. Finally, cells were washed with chilled vPBS and visualized under a Zeiss LSM 510 META confocal microscope.

**Generation of Rab5a and Rab5b Knock-out *L. donovani***—Attempts were made to generate LdRab5a and LdRab5b knock-out cells using pGL345-Hyg plasmid as described previously (31). Upstream regions flanking *Ldrab5a* were PCR-amplified (856 bp) using primers (forward, 5'-GCAAGCTTCCTCCGACTCCGCTTCCCG-3', and reverse, 5'-GCGTGCAGCTTTGTAGGTGATGTCGCGTTTTTCGGTG-3') from *L. donovani* genomic DNA as template. Similarly, downstream regions flanking *Ldrab5a* were amplified (1131 bp) using the following primers: forward, 5'-GCCCGGGTCCCCTCTCCGGACTACCTTCGTG-3', and reverse, 5'-GCAGATCTTGCTGGCAACATGGCACTGCCTTC-3'; upstream forward, 5'-GCAAGC-TTGGCACGAGGAAACCGTGACATG-3', and reverse, 5'-GCGTGCAGCGGTAGTGCGGATAATCGAAGCGG-3'; and downstream forward, 5'-GCCCGGGATGGGTGCCACGGGGTGGT-3', and reverse, 5'-GCAGATCTAACAGCACGACAGCTACAGCG-3' regions flanking *Ldrab5b* similarly amplified (1118 and 1010 bp) using appropriate primers. The upstream and downstream flanking sequences of LdRab5a or LdRab5b were sequentially cloned into pGL345-Hyg vector in the indicated restriction sites to generate pGL345-LdRab5a-Hyg or pGL345-LdRab5b-Hyg constructs and subsequently digested with HindIII and BglII to prepare LdRab5a-Hyg (5326 bp) or LdRab5b-Hyg (5467 bp) targeting knock-out cassette. In addition, pGL345-LdRab5a-NeoorpGL345-LdRab5b-Neo construct was generated by replacing the Hyg cassette with Neo cassette in pGL345-LdRab5a-Hyg or pGL345-LdRab5b-Hyg constructs, respectively. Finally, these constructs were digested with HindIII and BglII to prepare LdRab5a-Neo (5104 bp) or LdRab5b-Neo (5245 bp) targeting knock-out cassette.

Subsequently, *Leishmania* promastigotes were transfected with 10 μg of the purified LdRab5a-Hyg or LdRab5b-Hyg targeting knock-out cassettes using standard protocol (27). Cells were allowed to recover in drug-free medium for 30 h at 23 °C. Subsequently, cells were plated onto M199 agar plates containing hygromycin (20 μg/ml), and single colonies were isolated. These clones were grown in M199 containing 10% FCS and hygromycin (10 μg/ml) at 23 °C. Cells were harvested and washed, and genomic DNAs were prepared from untransfected LdRab5a-Hyg and LdRab5b-Hyg-transfected cells using genomic DNA purification kit (Promega). Subsequently, the genomic level of *rab5a* or *rab5b* was determined from each sample by quantitative PCR using LdRab5a- or LdRab5b-specific primers and SYBR Green Master Mix in a 7500 Fast Real Time PCR System (Applied Biosystems). *gapdh* was used as an internal control. The results were analyzed by the comparative Ct method (2<sup>-ΔΔCt</sup>) and are expressed as relative quantity of respective genes in different cell types. The clones from LdRab5a-Hyg- and LdRab5b-Hyg-transfected cells showing 50% reduction in the respective gene level indicated the generation of LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells after the first round of transfection. LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells were further transfected with LdRab5a-Neo and LdRab5b-Neo targeting knock-out cassettes, respectively, using the same protocol to generate LdRab5a and LdRab5b null-mutants. However, no viable cells were obtained after second round of transfection with LdRab5a-Neo or LdRab5b-Neo targeting knock-out cassettes.

**Identification of Putative LdRab5a and LdRab5b Interacting Partners by Mass Spectrometry**—Equimolar amounts of GST-LdRab5a (5 μg), GST-LdRab5b (5 μg), or GST (2.5 μg) were immobilized on glutathione-Sepharose beads and incubated with 3 mg of *Leishmania* lysate in 1.5 ml of PBS containing 0.2% Triton X-100 for 2 h at 23 °C. The beads were washed three times with PBS containing 0.1% Triton X-100 followed by three washes with PBS to remove unbound proteins. The bound proteins were resuspended in 100 μl of digestion buffer (50 mM ammonium bicarbonate solution containing 1 mg/ml trypsin gold) and incubated for 20 h at 37 °C. The beads were separated by centrifugation (2 min at 1000 × g), and the supernatant containing digested peptides was acidified using trifluoroacetic acid. The acidified digested peptides were concentrated to 50 μl and desalted using C-18 Zip-Tip by standard protocol. The digested peptides were vacuum-dried, dissolved in solvent A (5% acetonitrile containing 0.1% formic acid), and loaded for reverse phase chromatography using C-18 Picofrit analytical column in a Thermo-Scientific Proxeon Nano LC. Samples were run at a flow rate of 300 nl/min using a linear gradient of solvent B (95% acetonitrile containing 0.1% formic acid): 70 min in 5–40% solvent B, 10 min in 40–80% solvent B, 10 min in 80% solvent B, 5 min in 80–5% solvent B followed by 25 min in 5% solvent B. Mass spectrometry was performed in an Orbitrap Velos mass spectrometer, and data were analyzed using Thermo Proteome Discoverer Software (1.3.0.339 DBV version). Spectra of peptides were queried against *L. major* Uniprot database containing decoy database using a target false discovery rate of 1% for strict and 5% for relaxed conditions.



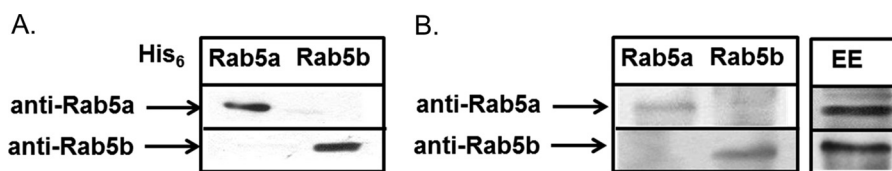


FIGURE 2. Specificities of anti-LdRab5a and anti-LdRab5b antibodies. *A*, specificities of anti-LdRab5a and anti-LdRab5b antibodies were determined using purified His<sub>6</sub>-LdRab5a and His<sub>6</sub>-LdRab5b proteins by Western blot analysis. *B*, Western blot analysis was carried out using lysates from LdRab5a- or LdRab5b-overexpressing *Leishmania* by anti-LdRab5a and anti-LdRab5b antibodies (*left panel*). Purified endosome containing avidin-HRP or biotinylated-Hbs were probed with anti-LdRab5a or anti-LdRab5b antibodies, respectively (*right panel*). All results are representative of three independent observations.

and 30% with *Trypanosoma*, human, and *Plasmodium* Rab5a sequences, respectively (Fig. 1*B*). Interestingly, the LdRab5a sequence showed an insertion of 21 amino acids (RGAAAAPG-GATSAHALQQMRA) in the loop2- $\beta$ 2 region in comparison with human. Previously, we cloned and characterized a Rab5b homolog (LdRab5b) from *Leishmania* (26), which showed about 74, 61, and 60% similarities with the *Trypanosoma*, human, and *Plasmodium* Rab5b sequences, respectively. The comparison of LdRab5a and LdRab5b sequences showed that both proteins contain five distinct Rab family motifs (RabF1-RabF5) and Rab5-specific subfamily motifs (SF1-SF4 regions) indicating these are Rab5 isoforms (32) from *Leishmania*. Rab5 isoforms in mammalian cells were shown to be more than 80% similar; however, LdRab5a and LdRab5b showed 62% similarity indicating that they are probably functionally more divergent (Fig. 1*C*).

Specific antibodies against LdRab5a and LdRab5b were made, and Western blot analysis showed that antibodies against LdRab5a specifically recognized His<sub>6</sub>-LdRab5a, whereas anti-Rab5b only detects His<sub>6</sub>-LdRab5b (Fig. 2*A*). These antibodies also specifically recognized respective proteins from LdRab5a- or LdRab5b-overexpressing *Leishmania* lysate (Fig. 2*B*, *left panel*). However, these antibodies failed to detect the respective endogenous protein from *Leishmania* lysate probably due to lower content of endogenous proteins; nevertheless, respective proteins were specifically detected by these antibodies in purified early endosomes from *Leishmania* as they are enriched in these compartments (Fig. 2*B*, *right panel*).

**Characterization of LdRab5 Isoforms from *L. donovani***—To determine the role of LdRab5 isoforms in endocytosis in *Leishmania*, dominant-active and dominant-negative mutants of LdRab5 isoforms were generated based on the similar mutations in mammalian Rabs (33). The LdRab5a:N146I mutant was made by substituting isoleucine for asparagine in the NKXD motif, whereas leucine was substituted for glutamine in the WDTAGQE region in LdRab5a:Q93L. Our results demonstrated that both LdRab5a:WT and LdRab5a:Q93L bind with [ $\alpha$ -<sup>32</sup>P]GTP, and no GTP-binding was observed with LdRab5a:N146I (Fig. 3*A*). Analysis of the GTPase activity of these mutants revealed that LdRab5a:WT protein hydrolyze GTP to GDP, whereas LdRab5a:Q93L is unable to hydrolyze the bound GTP to GDP as expected (Fig. 3*B*). Similarly, our results showed that LdRab5b:WT and LdRab5b:Q80L bind GTP, but LdRab5b:Q80L is unable to hydrolyze the bound GTP (Fig. 3, *C* and *D*). No GTP binding was observed with LdRab5b:N133I (Fig. 3*C*).

**Localization of LdRab5a and LdRab5b in *L. donovani***—LdRab5a and LdRab5b were overexpressed in *L. donovani* as GFP fusion proteins and their localizations were determined by

confocal microscopy. Our results showed (Fig. 4*A*) that LdRab5a is predominantly colocalized with 5 min internalized Alexa Fluor-594-conjugated Hb (correlation coefficient 0.80) as well as with 10 min internalization of dextran-Texas Red (correlation coefficient 0.95), which primarily labeled EE. Similarly, LdRab5b was also found to be colocalized with 5 min internalized Alexa Fluor-594-conjugated Hb (correlation coefficient 0.94) and 10 min internalization of dextran-Texas Red (correlation coefficient 0.84) (Fig. 4*B*). Conversely, LdRab5a and LdRab5b were found to be clearly separated from Texas Red-conjugated BODIPY-ceramide-labeled Golgi (correlation coefficient 0.10 and 0.04) and FM4-64 labeled flagellar pocket (correlation coefficient 0.12 and 0.21). No colocalization of LdRab5a was observed with the LysoTracker Red-labeled late/lysosomal compartment (correlation coefficient 0.20). However, LdRab5b also showed partial colocalization (correlation coefficient 0.79) with LysoTracker Red-labeled perinuclear late compartment (Fig. 4*B*). In addition, both RFP-LdRab5a and GFP-LdRab5b were found to be colocalized (correlation coefficient 0.96) in the early endosomal compartment in *Leishmania* (Fig. 4*C*).

**LdRab5a Specifically Regulates Fluid-phase Endocytosis in *L. donovani***—To study the role of LdRab5a or LdRab5b in FPE in *Leishmania*, cells overexpressing LdRab5a or LdRab5b (Fig. 5*A*, *inset*) were incubated with different concentrations of HRP for 1 h at 23 °C and washed, and HRP activity associated with the cells was measured. Interestingly, overexpression of GFP-LdRab5a specifically stimulated HRP uptake about 1.5-fold in comparison with the control cells (Fig. 5*A*). However, no effect on HRP uptake was observed in GFP-LdRab5b-overexpressing cells compared with control cells (Fig. 5*A*). In addition, results presented in Fig. 5*B* show that HRP uptake in LdRab5a:Q93L-overexpressing *Leishmania* was found to be even higher than the cells expressing LdRab5a:WT. In contrast, about 40% inhibition of HRP uptake in comparison with control was observed in cells overexpressing LdRab5a:N146I when cells were incubated with 2 mg/ml HRP. This 40% inhibition of HRP uptake by LdRab5a:N146I-overexpressing *Leishmania* is very significant as a substantial amount of LdRab5a:N146I protein was found to be degraded (Fig. 5*B*, *inset*). These results are supported by the fact that Ser/Asn mutant of Rab5 (dominant-negative) of *Trypanosoma* is also not stable in parasites and is degraded.

To visualize the role of LdRab5a and LdRab5b in FPE in *Leishmania*, we analyzed the trafficking of dextran-Texas Red conjugate in the cells overexpressing LdRab5a:WT, LdRab5b:WT, or control cells by confocal microscopy. Cells were incubated with dextran-Texas Red conjugate for 10 min at 23 °C to label the early endosomal compartment, washed, and chased

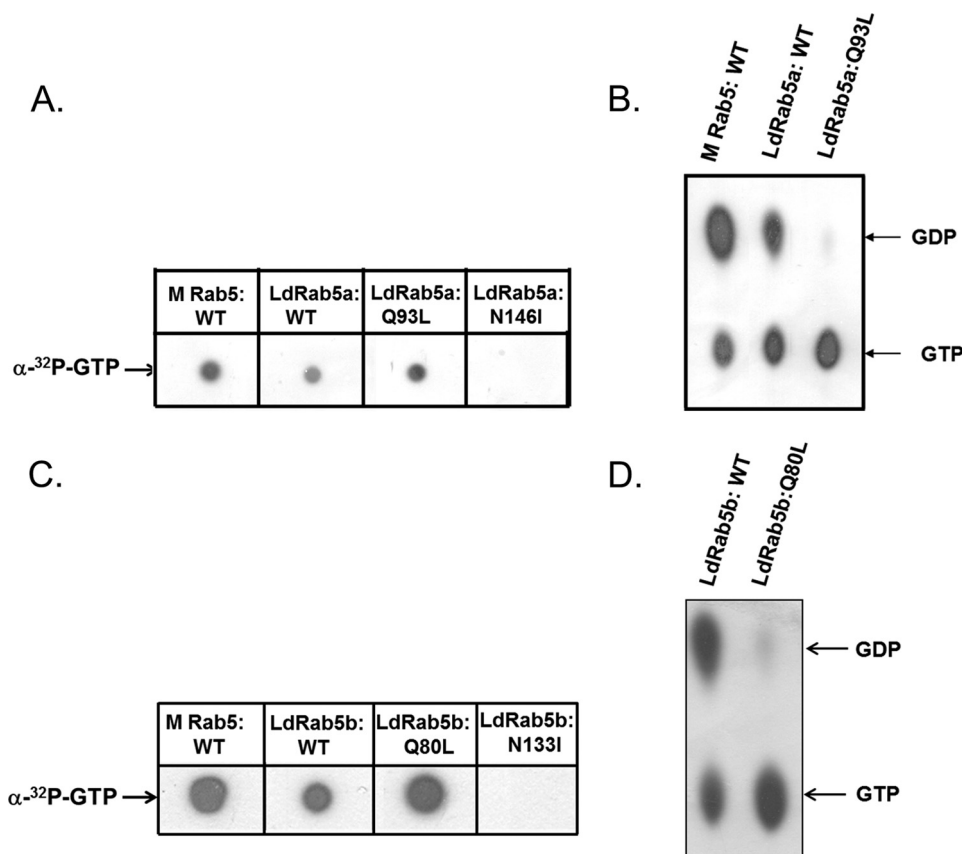
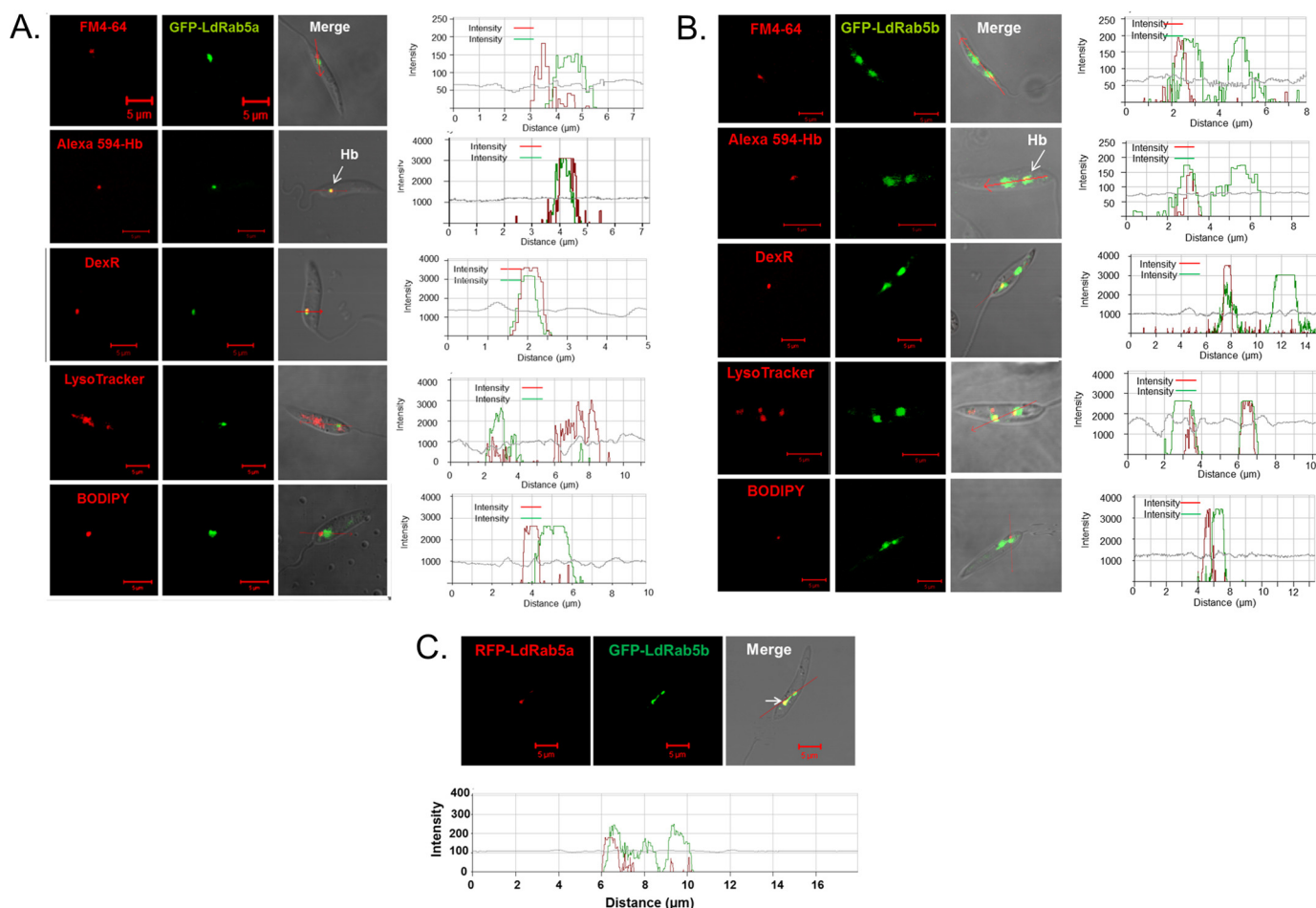


FIGURE 3. **Characterization of LdRab5 isoforms and their mutants.** GTP binding of the indicated purified proteins was detected using an [ $\alpha$ - $^{32}$ P]GTP overlay assay. *A*, LdRab5a and its mutants. *C*, LdRab5b and its mutants. GTPase activity of Rab5 isoforms was determined as described under "Experimental Procedures." *B*, LdRab5a and its mutants. *D*, LdRab5b and its mutants. Mammalian Rab5:WT was used as control. Results are representative of three independent observations.

for different time periods. Finally, localization of dextran-Texas Red with lysosomes stained with LysoTracker Green was determined as shown previously (34). Our results showed (Fig. 5C) that 10 min post-internalization of dextran-Texas Red is predominantly present in the early endosomal compartment by more than 90% in all cell types. Further analysis revealed that dextran-Texas Red reaches the perinuclear late endosomal compartment by about 30–60 min post-internalization, and complete colocalization of dextran-Texas Red with lysosomes (Fig. 5C, left) was observed at 90 min in control cells (correlation coefficient 0.82). In contrast, most of the internalized dextran-Texas Red was found in LysoTracker Green-labeled lysosomes within 60 min in LdRab5a-overexpressed *Leishmania* (correlation coefficient 0.94). However, internalized dextran was found to be accumulated in the lysosomes of LdRab5a-overexpressing *Leishmania* at about 90 min as dextran is not efficiently degraded in the lysosomes. These results demonstrated that LdRab5a not only induces FPE, but it also enhances the kinetics of lysosomal transport in comparison with control cells (Fig. 5C, middle). However, no significant difference in dextran-Texas Red trafficking was observed in LdRab5b-overexpressed cells compared with control cells (Fig. 5C, right). Further quantitative analysis of several micrographs ( $n = 50$ ) revealed (Fig. 5D) that dextran-Texas Red is colocalized with LysoTracker Green-labeled lysosomes in about 80% of Rab5a-overexpressed cells after 60 min of internalization in compari-

son with about 25% in control cells and 14% in LdRab5b-overexpressed cells. However, more than 80% of the control cells showed the colocalization of dextran-Texas Red with LysoTracker Green after 90 min of internalization in comparison with only about 26% in LdRab5b-overexpressed cells. Taken together, these results demonstrated that LdRab5a specifically regulates FPE in the parasite.

*LdRab5b Specifically Regulates Receptor-mediated Endocytosis of Hb in L. donovani*—Similar experiments were carried out to determine the role of LdRab5a or LdRab5b in RME in *Leishmania* promastigotes using Hb as an endocytic probe. In correlation with our previous finding, Hb uptake reached a steady state plateau after 3 h, whereas TCA-soluble radioactivity continued to increase at a linear rate (Fig. 6A), indicating simultaneous uptake and degradation of  $^{125}$ I-Hb by different transgenic *Leishmania*. Interestingly, our results (Fig. 6A) showed that LdRab5b-overexpressing cells induce almost a 30% increase in  $^{125}$ I-Hb uptake, whereas no significant change in the  $^{125}$ I-Hb uptake is observed in LdRab5a-overexpressing cells in comparison with control cells (Fig. 6A, left). Similarly, LdRab5b-overexpressing cells specifically showed about 2-fold higher degradation of Hb than control cells indicating that overexpression of LdRab5b enhances the degradation of internalized Hb (Fig. 6A, right). In contrast, degradation was found to be almost similar in LdRab5a-overexpressing cells and control cells. In addition, results presented in Fig. 6B showed that



**FIGURE 4. Intracellular localization of LdRab5a and LdRab5b in *L. donovani*.** *L. donovani* overexpressing GFP-LdRab5a (A) and GFP-LdRab5b (B) were stained with various compartment-specific markers, like FM4-64 (16  $\mu\text{M}$ ) for flagellar pocket, 5-min internalized Alexa Fluor-594 Hb, and 10-min internalization of dextran-Texas Red for early endosome, LysoTracker Red (100 nM) for lysosome, and Bodipy-TR ceramide (500 nM) for Golgi. Finally, cells were visualized under a confocal microscope. Results are representative of three independent observations. Arrow indicates colocalization. Red arrow indicates the intensities of various fluorescence probes in different regions. C, overexpression of RFP-LdRab5a and GFP-LdRab5b in *Leishmania* to determine the colocalization. All results are representative of three independent observations.

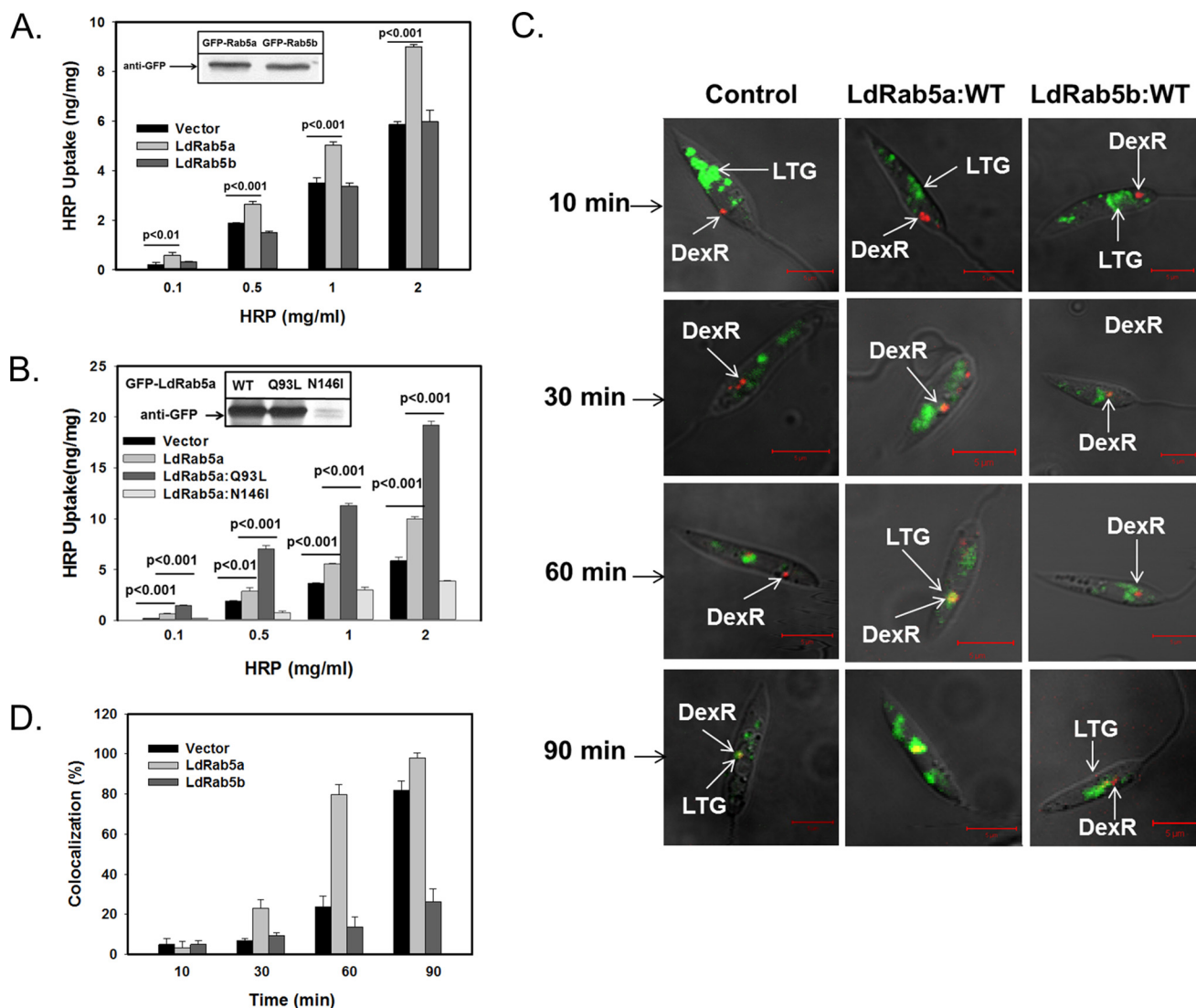
overexpression of LdRab5b:WT or LdRab5b:Q80L in *Leishmania* significantly induces both uptake and degradation of Hb in comparison with control cells. No significant differences in the Hb uptake and degradation were observed in cells overexpressing LdRab5b:N1331 mutant in comparison with control cells because most of the mutant protein was degraded inside the cells (Fig. 6B, inset).

To visualize the role of Rab5 isoforms in Hb trafficking in *Leishmania*, cells were incubated with Alexa 594-Hb for 5 min at 23  $^{\circ}\text{C}$  followed by a chase for different time periods. Subsequently, lysosomes were labeled with LysoTracker Green to determine the kinetics of Hb transport from early endosome to the lysosomes. Our results demonstrated (Fig. 6C, left) that Alexa 594-Hb is internalized into an early compartment near the kinetoplast by 15 min and subsequently moved to a perinuclear late endosomal compartment by about 30 min. Alexa 594-Hb was found to be colocalized with LysoTracker Green-labeled lysosomes at around 45 min (correlation coefficient 0.73), although no Hb was detected by 60 min of Hb internalization in control cells indicating the degradation of internalized Hb. Hb was detected in the perinuclear late endosomal compartment even after 60 min of internalization in *Leishma-*

*nia*-overexpressing LdRab5a (Fig. 6C, middle) demonstrating that overexpression of LdRab5a reduces the kinetics of Hb trafficking to the lysosomes in *Leishmania*. In contrast, Alexa 594-Hb was found to be colocalized with LysoTracker-labeled lysosomes at 30 min (correlation coefficient 0.96), and complete degradation of internalized Hb was detected by 45 min in LdRab5b-overexpressing cells (Fig. 6C, right). Further quantitative analysis of several micrographs ( $n = 50$ ) showed (Fig. 6D) that Alexa 594-Hb is colocalized with LysoTracker Green-labeled lysosomes in about 80% of LdRab5b-overexpressed cells after 30 min of internalization in comparison with less than 20% in control and LdRab5a-overexpressed cells. However, more than 85% of the control cells showed the colocalization of Alexa 594-Hb with LysoTracker Green after 45 min of internalization, whereas no Hb was detected in more than 95% in LdRab5b-overexpressed cells. In contrast, about 50% of LdRab5a-overexpressed cells showed colocalization after 60 min of Hb internalization, whereas internalized Hb is degraded in more than 95% of the control cells. These results demonstrated that LdRab5b in *Leishmania* not only induces Hb uptake but also enhances the targeting of internalized Hb to the lysosomes.



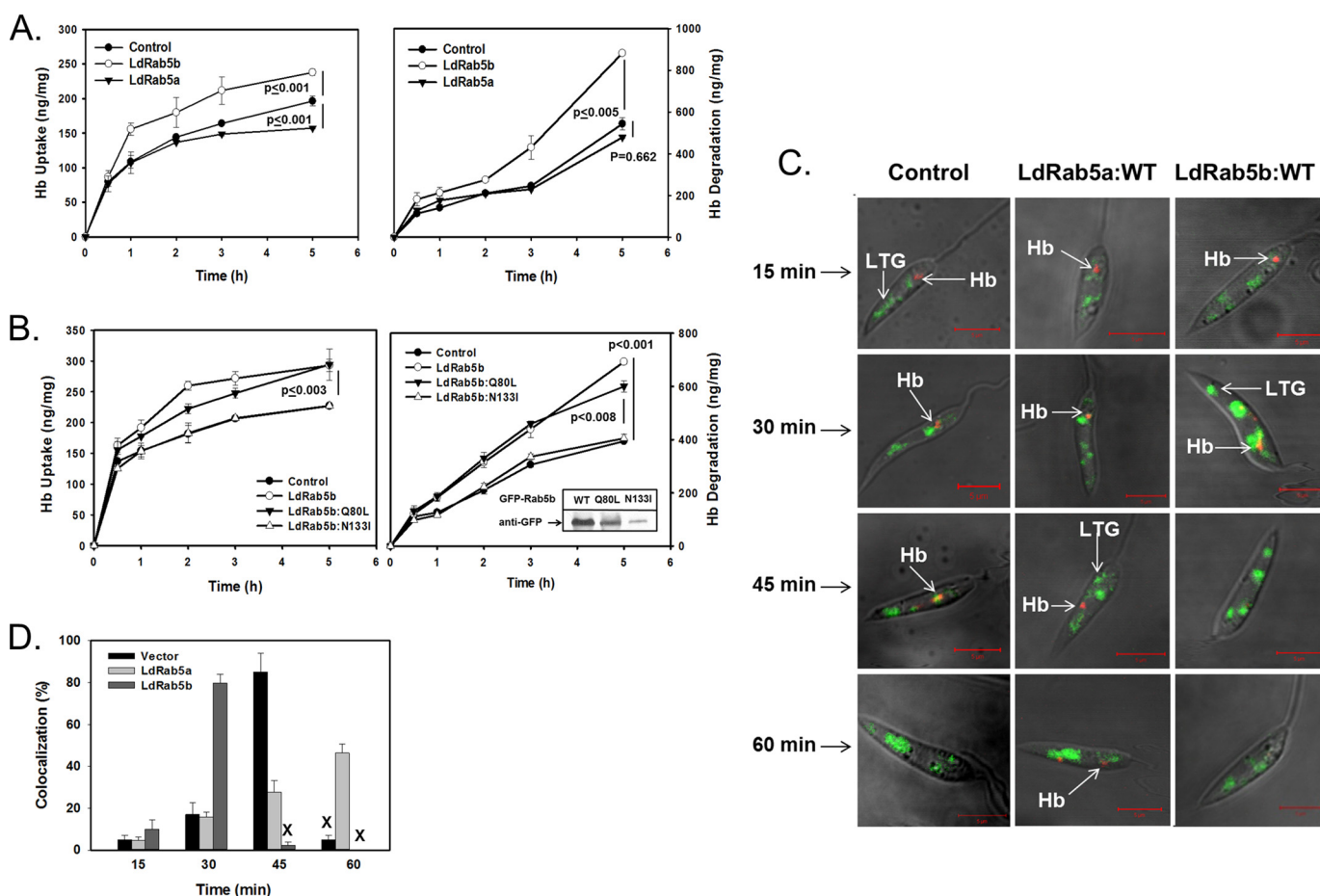
## Role of Rab5 Isoforms in Endocytosis



**FIGURE 5. LdRab5a specifically regulates fluid-phase endocytosis in *L. donovani*.** *A*, HRP was used as a probe to measure the fluid phase uptake in cells overexpressing GFP-LdRab5a or GFP-LdRab5b. The cells ( $2 \times 10^7$ ) were incubated for 1 h at 23 °C with the indicated concentrations of HRP, washed, and solubilized in solubilization buffer. An aliquot was used to measure the HRP activity from each sample and are expressed as nanograms of HRP uptake by respective cells  $\pm$  S.D. of three independent experiments. *Inset* shows the levels of expression of both proteins in respective cells. *B*, similar experiments were carried out using cells overexpressing GFP-LdRab5a:WT, GFP-LdRab5a:Q93L, or GFP-LdRab5a:N146I. Results are expressed as nanograms of HRP uptake by respective cells  $\pm$  S.D. of three independent experiments. *Inset* shows the levels of expression of each protein in respective cells. *C*, determination of kinetics of fluid phase endocytosis of dextran-Texas-red by *L. donovani*. Cells were incubated with dextran-Texas-red for 10 min at 23 °C, washed and chased for indicated period of times at 23 °C. The lysosomal compartments of these cells were stained with LysoTracker Green. Finally, cells were visualized under confocal microscope. *Yellow* indicates colocalization. Results are representative of three independent observations. *D*, quantitative analysis of percentage of cells showing the colocalization of dextran-Texas-red with LysoTracker Green in different cell types at indicated time points. Results are expressed as mean  $\pm$  S.D.

**Generation and Characterization of Rab5a and Rab5b Knock-out *L. donovani***—To determine the role of LdRab5a and LdRab5b in various modes of endocytosis in *Leishmania*, attempts were made to generate Rab5a and Rab5b knock-out *Leishmania* by targeted gene replacement (31) using appropriate gene knock-out cassettes (Fig. 7A). Essentially, the first copy of the *Ldrab5a* or *Ldrab5b* was replaced with the hygromycin resistance gene (*HYG*, encoding hygromycin phosphotransferase), and single knock-out cells were selected in semisolid medium in the presence of hygromycin. Genomic DNAs were isolated from hygromycin-resistant cells, and quantitative PCR was performed using gene-specific forward and reverse primers. The results presented in Fig. 7B showed about 50% reduction ( $2^{-\Delta\Delta Ct}$ ) in the level of respective Rab5 in single knock-out

cells in comparison with WT *Leishmania* demonstrating successful deletion of the single allele of the indicated gene to generate LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells. These results were further confirmed by RT-PCR (Fig. 7C). These cells were analyzed for correct integration of the hygromycin gene-targeting construct to one of the alleles of *Ldrab5a* or *Ldrab5b* by PCR using respective primers. As expected, 2309- and 2249-bp fragments were amplified from LdRab5a<sup>+/-</sup> and LdRab5b<sup>+/-</sup> cells, respectively (Fig. 7D). Subsequently, attempts were made to replace the second allele of LdRab5a<sup>+/-</sup> or LdRab5b<sup>+/-</sup> cells with the neomycin resistance gene cassettes (Neo, encoding neomycin phosphotransferase). Despite repeated attempts, we failed to obtain viable LdRab5a<sup>-/-</sup> and LdRab5b<sup>-/-</sup> cells indicating that functions of both LdRab5a and LdRab5b are



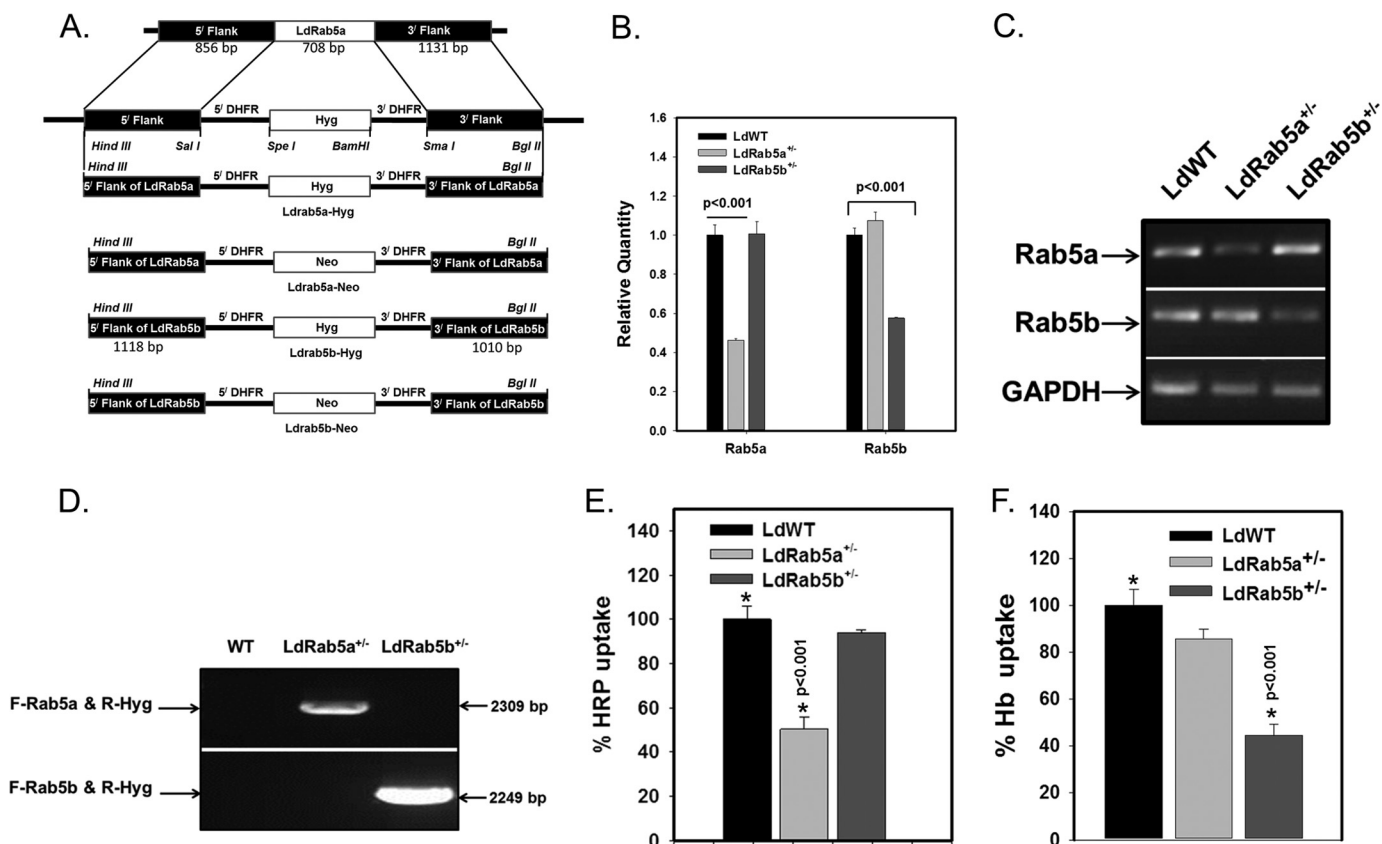
**FIGURE 6. LdRab5b specifically regulates the receptor-mediated endocytosis of Hb in *L. donovani*.** *A*, uptake (left panel) and degradation (right panel) of  $^{125}\text{I}$ -Hb was used to measure the receptor-mediated endocytosis of Hb in cells overexpressing GFP-LdRab5a or GFP-LdRab5b. The cells were incubated with  $^{125}\text{I}$ -Hb ( $6\ \mu\text{g}$ ) for the indicated times at  $23\ ^\circ\text{C}$ . The uptake and degradation of Hb were measured as mentioned under "Experimental Procedures." Vector-transfected cells were used as control. Results are expressed as nanograms of Hb per mg of cell protein  $\pm$  S.D. of three independent experiments. *B*, similar experiments were carried out using cells overexpressing GFP-LdRab5b:WT, GFP-LdRab5b:Q80L, or GFP-LdRab5b:N133I. Results are expressed as nanograms of Hb per mg of cell protein  $\pm$  S.D. of three independent experiments. *Inset* shows the levels of expression of each protein in respective cells. *C*, determination of the kinetics of receptor-mediated endocytosis of Hb in LdRab5a- or LdRab5b-overexpressing cells. Cells were incubated with Alexa-594-Hb for 5 min at  $23\ ^\circ\text{C}$ , washed, and chased for the indicated period of time at  $23\ ^\circ\text{C}$ . The lysosomal compartments of these cells were stained with LysoTracker Green. Finally, cells were visualized under a confocal microscope. *Yellow* indicates colocalization. Results are representative of three independent observations. *D*, quantitative analysis of percentage of cells showing the colocalization of Alexa-594-Hb with LysoTracker Green in different cell types at indicated time points. Results are expressed as mean  $\pm$  S.D. *X* indicates the complete degradation of internalized Hb in the respective cells.

possibly essential for parasites. Therefore, LdRab5a $^{+/-}$  and LdRab5b $^{+/-}$  cells were used to determine their role in various modes of endocytosis.

To unequivocally prove the role of LdRab5a and LdRab5b in FPE and RME in *Leishmania*, we compared the uptake of Hb and HRP in LdRab5a $^{+/-}$  and LdRab5b $^{+/-}$  cells. To determine the role of LdRab5a in FPE *Leishmania*, we measured the uptake of HRP by these cells and observed about 50% inhibition of HRP uptake in LdRab5a $^{+/-}$  in comparison with control cells (Fig. 7E). No significant change in HRP uptake was detected in LdRab5b $^{+/-}$  cells. Similarly, respective cells were also incubated with  $6\ \mu\text{g}/\text{ml}$  biotinylated Hb for 2 h at  $23\ ^\circ\text{C}$  and washed, and the amount of Hb present in the cell lysates after immunoprecipitating the internalized Hb with anti-Hb antibody was determined. Our results showed that about 50% of Hb uptake is specifically inhibited in LdRab5b $^{+/-}$  cells in comparison with control cells. No significant change in Hb uptake was observed in LdRab5a $^{+/-}$  cells (Fig. 7F).

*Mechanism of Regulation of Various Modes of Endocytosis in L. donovani by Rab5 Isoforms*—To understand the mechanism of regulation of FPE and RME in *Leishmania* by LdRab5a and LdRab5b, we tried to identify the putative interacting partners by mass spectrometry. Our interactome analysis showed (Fig. 8A) that 29 proteins might interact with both LdRab5 isoforms, whereas 32 and 26 proteins are specifically associated with LdRab5a and LdRab5b, respectively. However, most of the proteins were found to be unannotated and uncharacterized in *Leishmania*. Nonetheless, results presented in Table 1 showed some of the putative proteins that were found to be present in LdRab5a and/or LdRab5b interactomes. We found that some enzymes related to glucose metabolism, ATPases, kinases, motor proteins, etc. are present in LdRab5a and LdRab5b interactomes. Most importantly, our results also showed that hexokinase and clathrin heavy chain (LdCHC) are associated with the LdRab5b interactome. These results are consistent with our previous finding that the HbR is a hexokinase (25), and

## Role of Rab5 Isoforms in Endocytosis



**FIGURE 7. Generation and characterization of Rab5<sup>+/-</sup> and Rab5b<sup>+/-</sup> of *Leishmania*.** *A*, schematic representation of the generation of LdRab5a or LdRab5b gene knock-out constructs. *B*, genomic DNA was isolated from the indicated cloned cells, and DNA (35 ng) from respective cells was analyzed by quantitative PCR using LdRab5a- or LdRab5b-specific primers to validate *Leishmania*:WT, *Leishmania*:Rab5a<sup>+/-</sup>, and *Leishmania*:Rab5b<sup>+/-</sup>. Amplification of Ld-GAPDH by specific primers (forward, 5'-GAAGTACACGGTGGAGGCTG-3'; reverse, 5'-CGCTGATCACGACCTTCTTC-3') was used as a control. Results were analyzed with the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ) and are expressed as relative quantity of Rab5a or Rab5b. *C*, to determine the transcript levels of LdRab5a and LdRab5b, cDNA was prepared from indicated cells, and RT-PCR was carried out using gene-specific primers. GAPDH was used as control. *D*, to determine the correct integration of LdRab5a-Hyg or LdRab5b-Hyg targeting knock-out cassette to one of the alleles of the *LdRab5a* or *LdRab5b* gene, PCR was carried out using forward primer upstream to LdRab5a (forward, 5'-CATCGCAAGCGTCGGTGATGGGC-3') or LdRab5b (forward, 5'-CCTGTGCAGCCGCGCGCATTG-3') of the gene-targeting construct and reverse primer (reverse, 5'-CCGACGACATATCCACGCCCTC-3') specific for the hygromycin cassette from genomic DNA isolated from indicated cell types. Results are representative of three independent experiments. *E*, LdRab5a<sup>+/-</sup> cells specifically impair the fluid phase HRP uptake. *Leishmania*:WT, *Leishmania*:Rab5a<sup>+/-</sup>, and *Leishmania*:Rab5b<sup>+/-</sup> were incubated with 1 mg/ml HRP for 1 h at 23 °C, and HRP uptake by respective cells was measured as described previously. Results are represented as mean  $\pm$  S.D. of three independent experiments and expressed as relative percentage of HRP uptake by control cells, arbitrarily chosen as 100%. *F*, LdRab5b<sup>+/-</sup> cells specifically impair receptor-mediated uptake of Hb. *Leishmania*:WT, *Leishmania*:Rab5a<sup>+/-</sup>, and *Leishmania*:Rab5b<sup>+/-</sup> were incubated with 6  $\mu$ g/ml biotinylated Hb for 2 h at 23 °C, and Hb uptake by respective cells was measured as described under "Experimental Procedures." Results are represented as mean  $\pm$  S.D. of three independent experiments and expressed as relative percentage of Hb uptake by control cells, arbitrarily chosen as 100%.

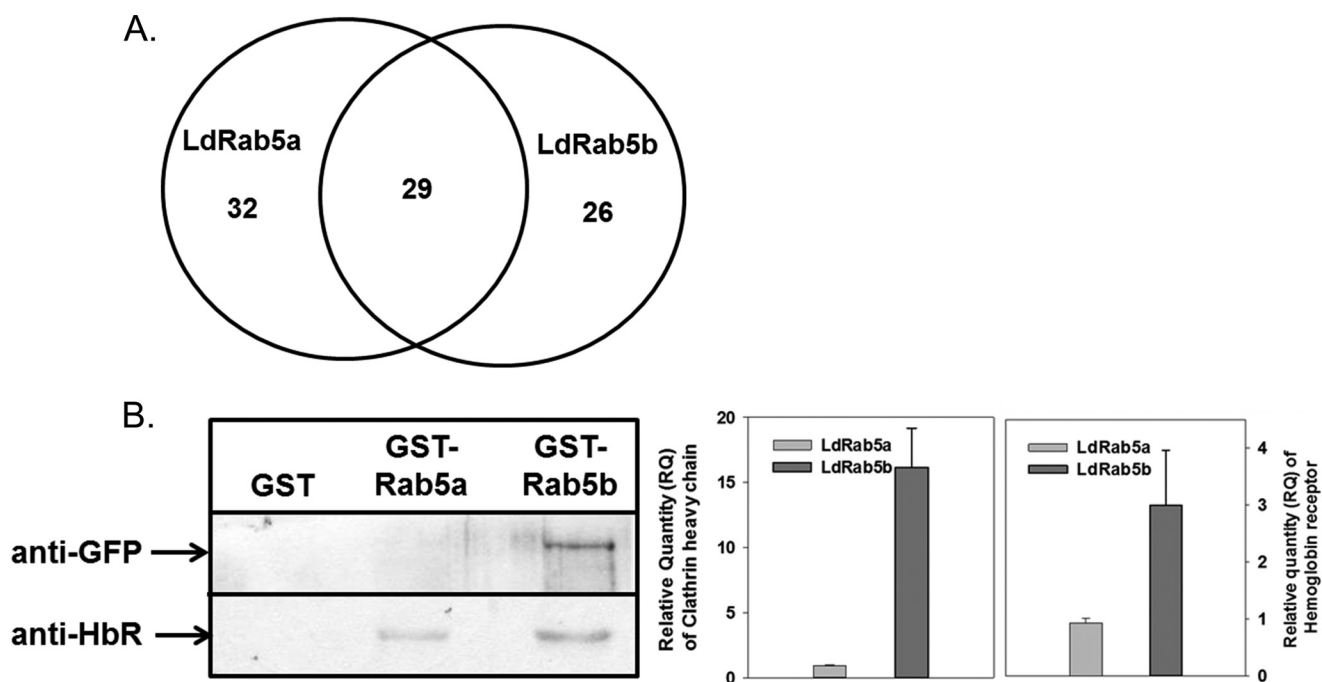
Hb endocytosis is a clathrin-dependent process (23) in *Leishmania*. Subsequently, we validated these observations using pull-down assay. Our results showed that immobilized GST-LdRab5b specifically binds with LdChc from GFP-LdChc-overexpressing *Leishmania* lysate. Similarly, more pronounced binding of LdRab5b was detected with HbR in comparison with LdRab5a (Fig. 8B).

### Discussion

In receptor-mediated endocytosis and fluid-phase endocytosis (4, 5), the endocytic ligands are internalized into Rab5-positive EE and subsequently are sorted to various intracellular destinations (35) like recycling (e.g. transferrin and low density lipoprotein) or degradation (e.g. epidermal growth factor). However, mechanism of sorting is not well characterized. Endosomal sorting could be more complex requiring several signaling molecules like phosphatidylinositol 3-kinase (36), receptor tyrosine kinase (37), and various effectors. Recent

studies have shown that APPL1, a Rab5 effector, regulates the differential sorting of EGF and transferrin from endosomes (38). Moreover, cargo sorting might occur at the plasma membrane depending on the mode of entry as clathrin-coated vesicles are also differentially tethered to the two types of endosomes (39). As all Rab5 isoforms are located on EE, it is tempting to speculate that Rab5 isoforms might have a differential role in regulating various modes of endocytosis.

In this study, we have used the unicellular parasite, *Leishmania*, because this parasite offers a very good model to study endocytosis and intracellular trafficking (23, 26, 27). Moreover, depletion, overexpression, or generation of the null-mutant of target gene/protein shows the phenotype and affects the viability of the cells and thereby indicates the essential functions of the respective Rabs in an organism. Previously, we have shown that Hb endocytosis is a clathrin-mediated process in *Leishmania* (23–25) and is essential for parasite survival (40). Therefore, we have used Hb as a receptor-mediated endocytic probe and



**FIGURE 8. Identification of putative effectors of LdRab5 isoforms by mass spectrometry.** *A*, to identify the LdRab5a- and LdRab5b-interacting partners, immobilized GST-LdRab5a or GST-LdRab5b was incubated with *Leishmania* lysate, and proteins bound with respective LdRab5 were analyzed by mass spectrometry as described under "Experimental Procedures." Venn diagram shows the number of proteins from *Leishmania* bind with indicated LdRab5. Results are representative of three independent experiments. *B*, binding of immobilized GST-LdRab5a and GST-LdRab5b with LdChC or HbR from GFP-LdChC-overexpressing *Leishmania* lysate as described under "Experimental Procedures." Western blot analysis was carried out using anti-GFP and anti-HbR antibodies. All results are representative of three independent observations.

**TABLE 1**  
Putative interacting partners for LdRab5a and LdRab5b identified by mass spectrometry

Results are representative of three independent observations and + denotes positive interaction. Accession numbers are from UniProt.

Accession no.	Putative protein	Rab5a	Rab5b
A4HUJ7	Nuclear transport factor 2	+	-
E9AGE0V	ATPase subunit F	+	-
A4HYX7	Coatamer subunit $\beta$	+	-
E9BA99	Mitogen-activated protein kinase 3	+	-
Q4QD34	Phosphoglycerate kinase	+	-
Q4Q6Z4	Glyceraldehyde- 3-phosphate dehydrogenase	-	+
A4HZB1	Hexokinase	-	+
K4HEE6	Clathrin heavy chain	-	+
Q4Q6S8	ATP synthase, $\epsilon$ chain	-	+
A4HLD6	$\beta$ -Tubulin	-	+
I3VJU6	Activated protein kinase C receptor	-	+
A4HUB4	Ribosomal protein I35a	-	+
A4HX43	Kinesin-like protein	-	+
A4I4E4	ADP ribosylation factor 3	-	+
A4I2L8	Acyl carrier protein	-	+
A4HSB4	Fructose-1,6-bisphosphatase	-	+
O77008	Casein kinase	-	+
A4HV09	Pyruvate phosphate dikinase	+	+
A4I6P8	Vacuolar-type proton translocating pyrophosphatase	+	+
A4I7I7	Dynein light chain	+	+
A4I9P1	Elongation factor 1- $\beta$	+	+

well characterized fluid-phase markers like HRP or dextran (41, 42) to study the role of Rab5 isoforms in different modes of endocytosis in *Leishmania*.

We have earlier reported that homotypic fusion between early endosomes is LdRab5-dependent in *Leishmania* (26). Further characterization reveals that previously reported LdRab5 is the LdRab5b isoform (43). Here, we have cloned, expressed, and characterized the LdRab5a homolog from *Leishmania*. Most importantly, both Rab5 isoforms of *Leishmania* contain

Rab5-specific subfamily motifs (32) and are predominantly localized in EE labeled with 5-min internalized Hb. Taken together, these results indicate that cloned proteins are *bona fide* Rab5 isoforms from *Leishmania*. It is important to mention that *Leishmania* has two isoforms of Rab5, whereas *Drosophila melanogaster* and *Caenorhabditis elegans* have only one Rab5 (44, 45), in contrast to three Rab5 isoforms in mammalian cells. These results indicate that *Leishmania* might have relatively better endosomal organization than *Drosophila melanogaster* and *Caenorhabditis elegans*. Previous studies have shown that TbRab5a and TbRab5b are predominantly associated near the flagellar pocket of *Trypanosoma* (43). Subsequently, it has been shown that TbRab5a and TbRab5b are differentially expressed in bloodstream form and procyclic form in *Trypanosoma*, and both are required for the endocytosis of several ligands like tomato lectin, Lucifer Yellow, and LDL in stage-specific parasites (46, 47). However, the role of Rab5 isoforms in specifically regulating FPE and RME in a particular stage of parasites is not yet depicted. We have recently shown that neither PbRab5a nor PbRab5c can complement for the loss of function of PbRab5b indicating their non-overlapping roles in *Plasmodium berghei* (48). Therefore, we have overexpressed LdRab5a and LdRab5b in *Leishmania* to determine their roles in various modes of endocytosis in parasites.

We have generated constitutively active and dominant-negative mutants of LdRab5a and LdRab5b and overexpressed them in *Leishmania* as GFP fusion proteins to determine their specific functions. Our results have shown that overexpression of GFP-LdRab5a:WT and LdRab5a:Q93L specifically induces the fluid-phase endocytosis of HRP and dextran-Texas Red,

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although no significant difference in HRP uptake was observed in GFP-LdRab5b-overexpressing *Leishmania* in comparison with control parasites. HRP uptake is found to be significantly inhibited in LdRab5a:N146I-overexpressing transgenic parasites even though substantial amounts of LdRab5a:N146I protein are degraded in these cells. This is consistent with the observation that the dominant-negative mutant of Rab5 is also degraded in *Trypanosoma* (46). We have failed to generate LdRab5a null-mutants despite repeated attempts demonstrating that LdRab5a function is essential in the parasite. Therefore, we have used heterozygous LdRab5a<sup>+/-</sup> cells to study RME and FPE. Interestingly, we have found about 50% inhibition of HRP uptake specifically in LdRab5a<sup>+/-</sup> cells in comparison with WT:*Leishmania*. No significant difference in Hb endocytosis is observed in these cells. Taken together, these results demonstrate that LdRab5a specifically regulates FPE in the parasite.

In contrast, LdRab5b:WT- and LdRab5b:Q80L-overexpressing cells show enhanced uptake and degradation of Hb in comparison with control cells, but Hb endocytosis is unaffected in LdRab5a-overexpressing cells. To unequivocally prove the role of LdRab5b in RME in *Leishmania*, we have tried to generate LdRab5b null-mutant *Leishmania*. However, we have failed to generate the LdRab5b null-mutant despite repeated attempts indicating that LdRab5b function is also essential in the parasite. Therefore, we have used heterozygous LdRab5b<sup>+/-</sup> cells to study RME and FPE. Interestingly, we have found about 50% inhibition of Hb uptake specifically in LdRab5b<sup>+/-</sup> cells in comparison with WT:*Leishmania*. No significant inhibition of HRP uptake is observed in these cells. Taken together, these results demonstrate that LdRab5b specifically regulates RME of Hb in the parasite.

It has been shown in mammalian cells that depending on receptor-ligand interaction, ligand can be internalized into different populations of endosomes; for example, transferrin is internalized into EEA1-positive conventional EE; TGF $\beta$  is internalized into endosome enriched with the FYVE domain-containing protein SARA (49); and EGF receptors are shown to be internalized into APPL1-positive EE (50). This could be due to the interaction with different adaptor proteins (51). Similarly, it has been shown in *Trypanosoma* that TbRab5a is colocalized with endosomes containing variant surface glycoprotein and transferrin, whereas TbRab5b is specifically present in endosomes containing ISG100 speculating that different cargo in *Trypanosoma* might enter into different compartments (46). However, mechanism of sorting is not clearly established.

It will be interesting to define how these two modes of endocytosis are specifically regulated by Rab5 isoforms in *Leishmania*. It is well established that Rab5 regulates endocytic trafficking by interacting with several effectors in mammalian cells, e.g. guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), etc. Surprisingly, at least six different Rab5 GEFs like Rabex5, Gapex-5, Rin1, Rin2, Rin3, and Als2 are known today in mammalian cells (52–55). Similarly, RabGap-5 and RN-Tre are Rab5-associated GAPs (56, 57). Thus, the possibility of one Rab5 isoform associated with one specific GEF, GAP, or effector cannot be ruled out. This is supported by the

facts that Rin1 selectively activates Rab5a (12), whereas early endosome antigen 1 (EEA1) interacts with Rab5b (58). Thus, it is tempting to speculate that LdRab5 isoforms might interact with specific effectors to regulate a particular mode of endocytosis in *Leishmania*. However, effectors of different Rabs in trypanosomatid parasites are not yet characterized. We tried to identify the putative interacting partners by mass spectrometry. Surprisingly, our LdRab5a and LdRab5b interactome data do not show any of the conventional Rab5 effectors reported in mammalian cells like EEA1, Rabex5, Rabaptin5, and Rabenosyn5. Moreover, a search for these conventional Rab5 effectors in the *L. major* database using mammalian Rab5 effectors as query sequences does not show any homologs of these proteins in *Leishmania*. However, our LdRab5 interactome data predicted a large number of proteins that might interact with LdRab5a and/or LdRab5b, but most of them are unannotated and uncharacterized. Moreover, signal transduction as well as protein trafficking pathways in *Leishmania* are not well characterized; therefore, the identification of effector molecules is more challenging in parasites. However, we have found some LdRab5 interactors like dynein, kinesin, and tubulin that are shown to regulate endocytosis and vesicular trafficking (59, 60). Moreover, Rab5 is also reported to transport cargo to the nucleus (61); thus, interaction with a nuclear transport factor is an interesting observation. Interestingly, LdRab5b interactome data suggest that this protein might interact with glycolytic enzymes like hexokinase and glyceraldehyde 3-phosphate dehydrogenase. This is possible as the Hb receptor in *Leishmania* is a hexokinase (25). In addition, cell surface-associated glyceraldehyde-3-phosphate dehydrogenase is reported to be involved in iron acquisition in *Staphylococcus* (62), and the ATP synthase subunit is found to be an HDL receptor in hepatocytes (63). Moreover, in correlation with our findings, casein kinase-1 is reported to be a Rab5b effector in *Plasmodium* (64). Our results have also validated that LdRab5b specifically interacts with LdChc and HbR.

The possibilities of LdRab5 isoforms interacting with different effectors are also supported by the facts that LdRab5a contains an insertion of 21 amino acids (RGAAAAPGGATSAHALQQMRA) in the loop2- $\beta$ 2 region, so it might interact with specific effector(s) to regulate the FPE in *Leishmania*. LdChc of *Leishmania* specifically contains an insertion of 10 amino acid residues (MAQKQDTDLN) in the distal domain (23) and also interacts with LdRab5b, suggesting that LdRab5b-regulated clathrin-mediated endocytosis might be controlled by different effector(s). Taken together, these results suggest that LdRab5b plays a major role in regulating receptor-mediated endocytosis. However, LdRab5a failed to interact with LdChc thus possibly driving to fluid-phase endocytosis.

In conclusion, our results represent the first demonstration that Rab5 isoforms differentially regulate fluid-phase and receptor-mediated endocytosis in *Leishmania*. We speculate that regulation of different modes of endocytosis by specific Rab5 isoforms could be due to their interaction with different effectors. Currently, we are trying to identify and understand the role LdRab5a- and LdRab5b-specific effectors in the regulation of various endocytic pathways in parasites.

**Author Contributions**—A. M. conceived and coordinated the study and wrote the paper. R. R., J. K. V., and A. K. performed the experiments and analyzed the results. G. L. did the bioinformatics and analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

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