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Rab5-mediated endosome–endosome fusion regulates hemoglobin endocytosis in *Leishmania donovani*

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To understand the trafficking of endocytosed hemoglobin (Hb) in *Leishmania*, we investigated the characteristics of *in vitro* fusion between endosomes containing biotinylated Hb (BHb) and avidin–horseradish peroxidase (AHRP). We showed that early endosome fusion in *Leishmania* is temperature and cytosol dependent and is inhibited by ATP depletion, ATPγS, GTPγS and *N*-ethylmaleimide treatment. The Rab5 homolog from *Leishmania donovani*, LdRab5, was cloned and expressed. Our results showed that homotypic fusion between the early endosomes in *Leishmania* is Rab5 dependent. Early endosomes containing BHb fused efficiently with late endosomes in a process regulated by Rab7, whereas no fusion between early and late endosomes was detected using fluid phase markers. Pre-treatment of early endosomes containing BHb with monoclonal antibody specific for the C-terminus of the Hb receptor (HbR) or the addition of the C-terminal cytoplasmic fragment of the HbR specifically inhibited the fusion with late endosomes, suggesting that signal(s) mediated through the HbR cytoplasmic tail promotes the fusion of early endosomes containing Hb with late endosomes.

Keywords: endocytosis/Hb/*Leishmania*/Rab/reconstitution

Introduction

Endocytosis is a fundamental process that mediates internalization, sorting and degradation of endocytosed molecules (Wileman *et al*., 1985). In protozoan parasites, endocytosis is mainly studied in trypanosomatids (Clayton *et al*., 1995; Liu *et al*., 2000), and a few receptor systems mediating efficient supply of nutrients have been identified (Voyiatzaki and Soteriadou 1992; Bastin *et al*., 1996). However, the intracellular route and mechanism of transport of internalized materials in these parasites remain largely unknown.

Recent studies have established that transport of cargo along the endocytic pathway requires a series of coordinated and specific vesicle fusion events regulated by small GTP-binding proteins of the Rab family (Zerial and McBride, 2001). Among the endocytic Rabs, Rab7 is involved in transport from the early to late compartment (Feng *et al*., 1995; Mukhopadhyay *et al*., 1997b), whereas Rab5 regulates transport from the plasma membrane to the early compartment as well as homotypic fusion among early endosomes (Gorvel *et al*., 1991; Mukhopadhyay *et al*., 1997a). Indications of similar endocytic and secretory pathways in trypanosomatids, reported recently (McConville *et al*., 2002), remain to be characterized. Homologs of Rab4, Rab5, Rab7 and Rab11 have been identified in *Trypanosoma brucei*. Rab4 and Rab11 appear to be involved in recycling, while different isoforms of Rab5 regulate distinct steps in endocytosis (Field and Field, 1997; Field *et al*., 1998; Pal *et al*., 2002). Rab7, and to a lesser extent Rab4, are associated with the endocytic pathway in *Dictyostelium discoideum* (Temesvari *et al*., 1994; Laurent *et al*., 1998). In *Toxoplasma gondii*, Rab5 regulates cholesterol acquisition from the host cell (Robibaro *et al*., 2002) and Rab6 is involved in sorting of post-Golgi secretory granules (Stedman *et al*., 2003). However, regulation of intracellular trafficking by Rab GTPases in protozoan parasites remains to be elucidated. Recently, we have shown that endocytosis of hemoglobin (Hb) in *Leishmania* is mediated through receptors located in the flagellar pocket (Sengupta *et al*., 1999), possibly to generate intracellular heme after degradation of internalized Hb, as *Leishmania* lack a complete heme biosynthetic pathway (Sah *et al*., 2002). In order to understand the regulation of Hb trafficking in *Leishmania*, we have reconstituted endosome fusions using endosomes containing Hb purified from *Leishmania* promastigotes. Here we report the cloning and expression of Rab5 from *L.donovani* and show that the early endosome in *Leishmania* is a very dynamic compartment and promotes fusion with early and late compartments during Hb trafficking.

Results

Cloning and expression of Rab5 homolog from L.donovani

To clone the Rab5 homolog from *L.donovani*, a BLAST (Karlin and Altschul, 1993) search was carried out using human Rab5 as a query. Genes from divergent genera of protozoa showed significant similarity with human Rab5, e.g. *D.discoideum* (88%), *T.gondii* (75%), *Plasmodium falciparum* (78%), *Entamoeba histolytica* (78%) and *T.brucei* (61%). As *Trypanosma* are closest to *Leishmania*, a second search was carried out using *T.brucei* Rab5B sequence as a query, which revealed a putative Rab5-like sequence from *L.major* with 72% homology. Putative start and stop codons were predicted and appropriate forward and reverse primers were used to

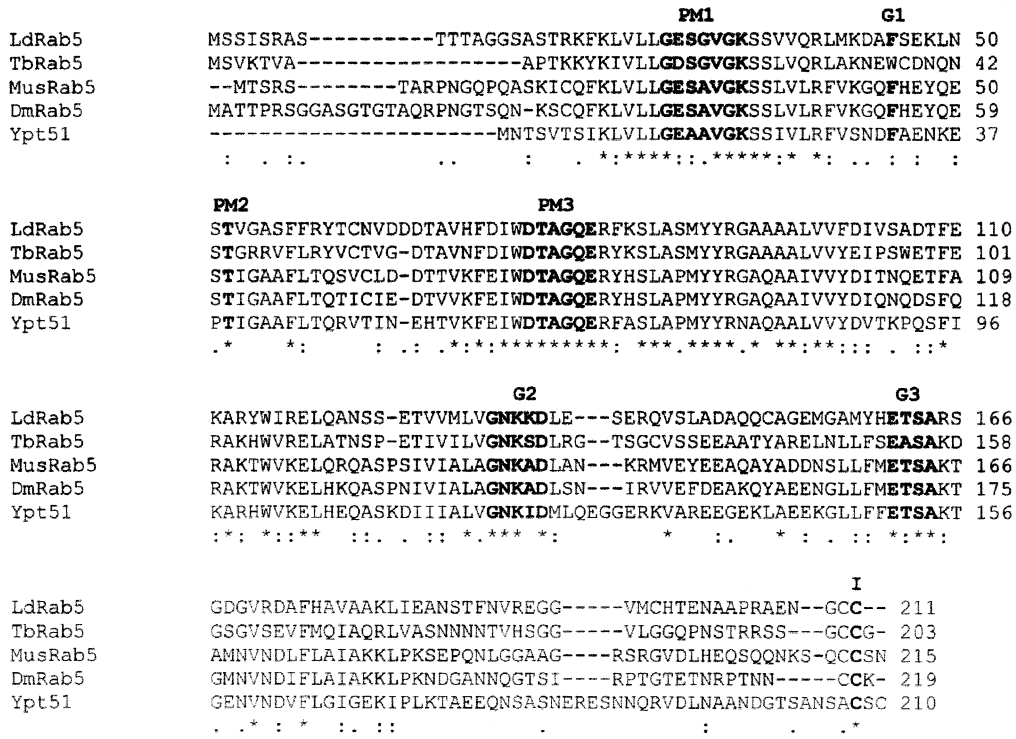

Fig. 1. Multiple alignment of the amino acid sequence of LdRab5 with Rab5 sequences from different organisms. Our LdRab5sequence data have been submitted to the GenBank database under accession No. AY337265. Residues implicated in guanine phosphate binding (PM1–3), GTP/GDP binding (G1–3) and the isoprenylation motif (I) are marked in bold. Tb, *T.brucei*; Mm, *M.musculus*; Dm, *D.melanogaster*; Ypt, *S.cerevisiae*; Hs, *H.sapiens*; Ld, *L.donovani*; Lm, *L.major*; Tg, *T.gondii*; Pf, *P.falciparum*.

amplify a fragment (636 bp) from *L.donovani* cDNA by PCR. The PCR product was cloned, sequenced and hypothetically translated (211 amino acids). A BLAST search revealed that the cloned protein (LdRab5) has ~91% similarities with *L.major* putative Rab5, 65% with *T.brucei* Rab5, 66% with *T.gondii* Rab5, 62% with *D.melanogaster* Rab5 and 59% with human and mouse Rab5. Comparison of LdRab5 sequence with other Rab5 sequences using ClustalW multiple sequence alignment (Version 1.8; Thompson *et al*., 1994) demonstrated the presence of conserved Rab protein features (Stenmark and Olkkonen, 2001) including the GTP-binding region, effector loop and C-terminal isoprenylation motif (Figure 1).

Characterization of Rab5 from L.donovani

GTP binding and GTPase activities of GST–LdRab5 were determined using free GST and mammalian GST–Rab5 as controls. Figure 2A shows that unlike free GST, GST–LdRab5 bound a significant amount of [α-^{32}P]GTP. The binding of [α-^{32}P]GTP was competed out by unlabeled GTP and GDP but not by ATP, indicating the specificity of guanine nucleotide binding of LdRab5 (Figure 2B). However, kinetic analysis of GTPase activity revealed that LdRab5 GTPase activity is comparatively reduced with respect to mammalian Rab5 (Figure 2C).

Specificity of antibodies against LdRab5 and LdRab7

Antibodies against LdRab5 and LdRab7 were raised to characterize Hb trafficking in *Leishmania*. Western blot analysis showed that antibodies against LdRab5 and LdRab7 specifically recognized an ~23 kDa and an ~27 kDa protein, respectively, from *Leishmania* lysate (Figure 3) and did not cross-react with GST (data not shown).

Subcellular fractionation and preparation of Rab5-enriched early endosomal compartment from Leishmania

Previously we showed that Hb is internalized efficiently into the endocytic compartment within 5 min in *Leishmania* (Sengupta *et al*., 1999). Thus, internalization of biotinylated Hb (BHb) for 5 min was used to label the endocytic compartment. Another set of endosomes was labeled by 5 min internalization of avidin–horseradish peroxidase (AHRP), a fluid phase marker (Gorvel *et al*., 1991). In order to characterize compartments labeled with AHRP, subcellular fractionation was carried out and HRP activity was estimated from different fractions (50 µl) collected from the top of the gradient. Maximum HRP activity was obtained at the 8–30% interface (Figure 4). No significant HRP activity was detected at the top of the gradient, indicating that most of the endosomes were intact. Five successive fractions were pooled, washed and solubilized in SDS buffer, and western blot analyses were carried out to determine the nature of the compartments. Results presented in Figure 4 show that Rab5 and transferrin receptor were predominantly present in fraction numbers 6–10, which retained maximum HRP activity, whereas a Rab7-positive compartment was found in the later fractions with relatively low HRP activity. The

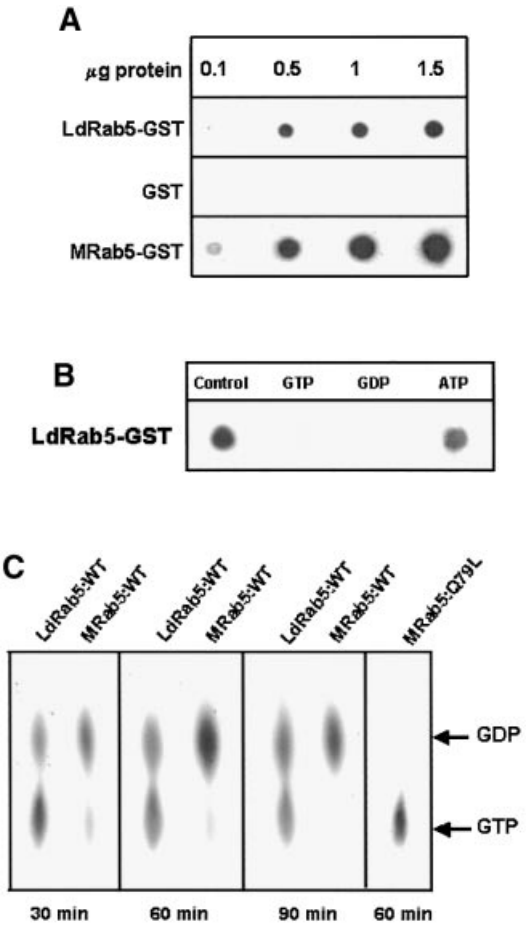

Fig. 2. Characterization of Rab5 from *L.donovani*. (**A**) GTP binding of the indicated concentrations of LdRab5 was detected using a [α-^{32}P]GTP overlay assay. Free GST and mammalian Rab5 were used as control. (**B**) [α-^{32}P]GTP binding was measured in the absence (control) or presence of 1 mM of the indicated nucleotides. (**C**) The kinetics of GTPase activity of LdRab5 were determined as described in Materials and methods. Mammalian Rab5 wild-type (WT) protein and Rab5:Q79L, a GTP locked mutant, were used as control. Results are representative of three independent preparations.

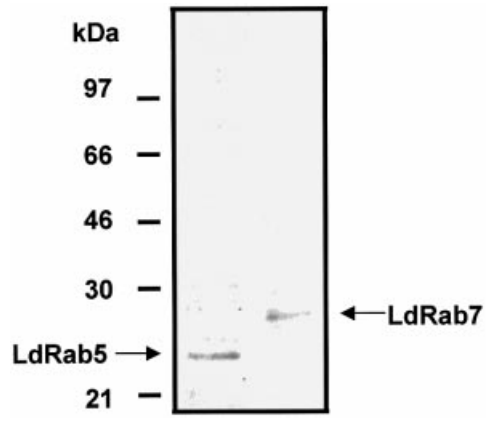

Fig. 3. Specificity of *Leishmania* Rab5 and Rab7 antibodies. *Leishmania* cell lysate (40 μg/lane) was analyzed by western blot using specific antibodies against LdRab5 and LdRab7. Results of western blots are representative of three independent preparations.

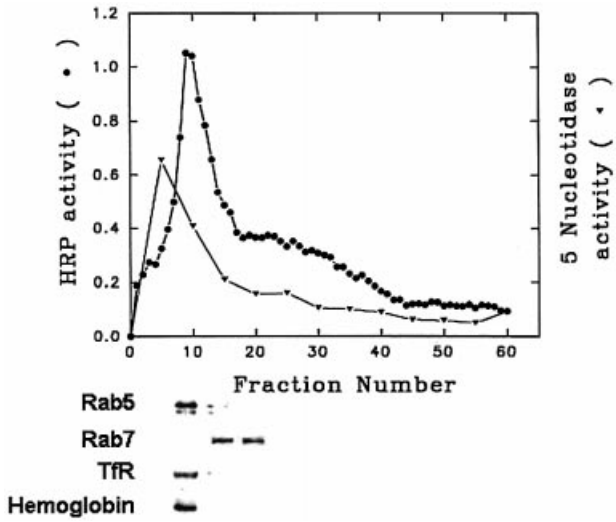

Fig. 4. Subcellular fractionation of the *L.donovani* endocytic compartment. Promastigotes were disrupted after internalization of AHRP, and PNS was loaded onto a discontinuous sucrose gradient as described in Materials and methods. After centrifugation, 50 μl fractions were collected from the top of the gradient and analyzed for the presence of HRP. Five successive fractions from the top of the gradient were pooled, washed and analyzed by western blot for the presence of the indicated proteins (TfR, transferrin receptor). 5′-Nucleotidase activity was also measured from the pooled fractions. Similar fractionation was carried out using BHb as a probe, and the presence of Hb in the fractions was analyzed by western blot using anti-Hb antibody. Results from western blots are representative of three independent preparations.

doublet observed for Rab5 might be isoforms or prenylated and non-prenylated protein. Maximum activity of 5′-nucleotidase, a plasma membrane marker, was detected in the lighter fractions having relatively lower HRP activity (Figure 4). Hb was also detected in the fractions enriched in Rab5 and transferrin receptor, when similar fractionation was carried out following 5 min internalization of BHb.

Reconstitution and characterization of in vitro endosome–endosome fusion in Leishmania

Reconstitution of endosome fusion has been used successfully to determine the requirements for endocytosis (Gorvel *et al*., 1991). The results presented in Figure 5A show a typical *in vitro* fusion experiment in which two sets of endosomes containing BHb or AHRP were incubated for 1 h at 23°C in the presence of an ATP-regenerating system containing different concentrations of gel-filtered cytosol prepared from *Leishmania* promastigotes. Maximum fusion between the endosomes was observed at 1 mg/ml cytosol concentration. No fusion was detected in the absence of cytosol, suggesting a role for the cytosolic components in endosome fusion. The extent of early endosome fusion in *Leishmania* was significantly more at 23°C than at 37°C, whereas no fusion was detected at 4°C (data not shown). To determine the energy requirement, endosome fusion was carried out in an ATP-depleting system [250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.2, 1 mM dithiothreitol (DTT), 1.5 mM $MgCl_2$, 100 mM KCl containing 5 mM glucose and 25 U/ml hexokinase, and 0.25 mg/ml avidin as scavenger]. Under these conditions, as well as in the

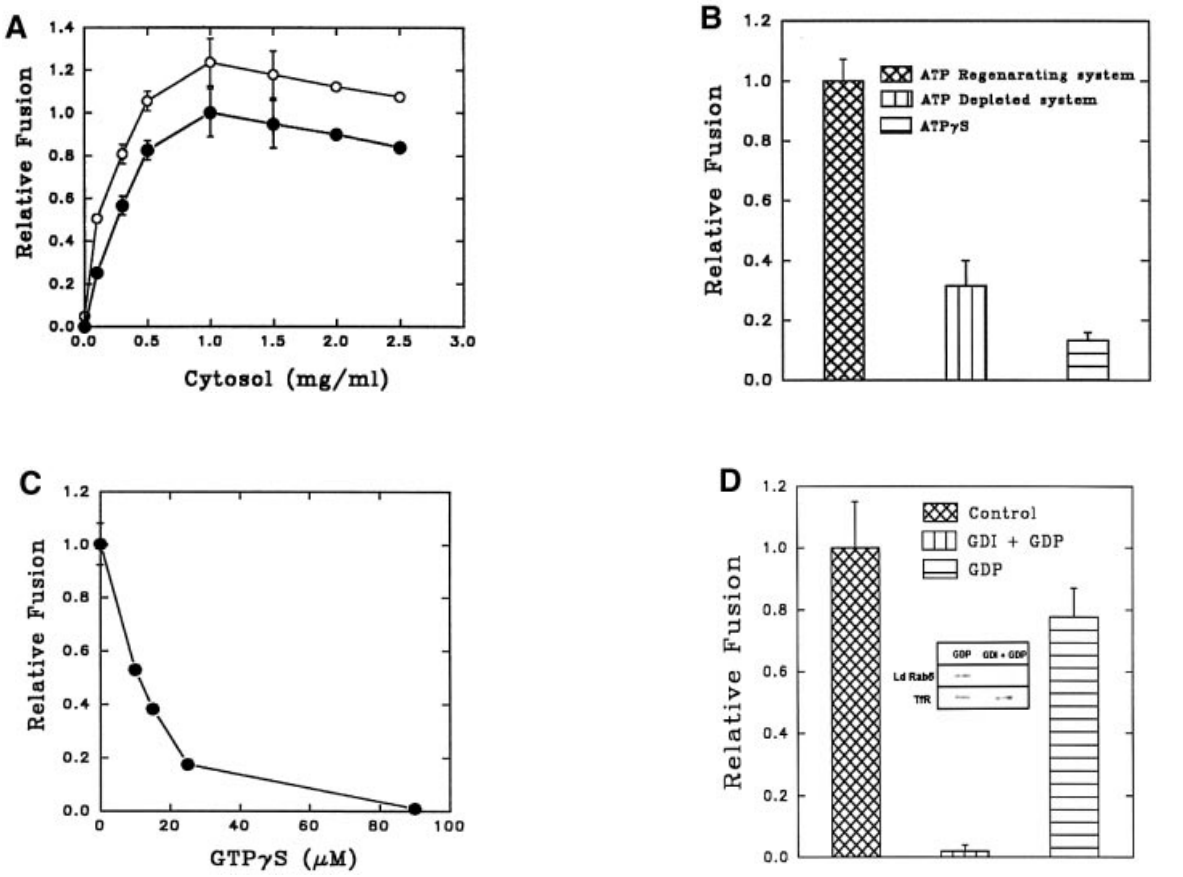

Fig. 5. Characterization of *in vitro* early endosome fusion in *Leishmania*. (**A**) Two sets of early endosomes containing BHb or AHRP were incubated in the presence of an ATP-regenerating fusion buffer supplemented with different concentrations of gel-filtered *Leishmania* cytosol for 1 h at 23°C. Specific fusion (filled circles) was measured by subtracting the value obtained in the absence of cytosol from the total fusion (open circles) observed in the presence of cytosol as indicated in Materials and methods. Maximum fusion was observed at 1 mg/ml of cytosol, which was normalized to 1 U, and results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 12 ng of HRP activity/mg of protein. (**B**) *In vitro* fusion between endosomes from *Leishmania* was carried out in an ATP-regenerating system (Control), ATP-depleted system or in the presence of ATPγS (4.5 μM). Fusion obtained in the control was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 13.6 ng of HRP activity/mg of protein. (**C**) Fusion between endosomes in *Leishmania* was carried out in an ATP-regenerating system in the presence of different concentrations of GTPγS and 1.5 mg/ml cytosol. Fusion obtained in the absence of GTPγS was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 12.6 ng of HRP activity/mg of protein. (**D**) Endosomes containing BHb treated either with GDP (1 mM) alone or with GDI (6 μg/ ml) as described (Mukherjee *et al*., 2000) were centrifuged and pellets were analyzed for the presence of Rab protein by western blot using anti-LdRab5. Transferrin receptor (TfR) was used as a control (inset). Treated and untreated (control) endosomes containing BHb were used in the fusion assay. Fusion obtained with untreated endosomes was chosen as 1 U, and results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 14.5 ng of HRP activity/mg of protein.

presence of a 4.5 μM ATPγS-containing ATP-regenerating system, significant inhibition of fusion was observed between endosomes, indicating that both ATP and ATP hydrolysis are required for this process (Figure 5B).

About 80% fusion between early endosomes at a high cytosol concentration (1.5 mg/ml) was inhibited by 25 μM GTPγS, a non-hydrolyzable analog of GTP (Figure 5C). Moreover, treatment of endosomes containing BHb with 6 μg/ml of mammalian GDI (GDP dissociation inhibitor) in the presence of 1 mM GDP specifically removed *Leishmania* Rab protein, i.e. Rab5, but not transferrin receptor (Figure 5D, inset). Rab-depleted endosomes containing BHb inhibited fusion with endosomes containing AHRP by ~90%, indicating that Rab GTPases may be regulating this fusion process (Figure 5D).

Role of NSF-like protein in endosome fusion in Leishmania

In vitro fusion between endosomes from mammalian cells is inhibited by *N*-ethylmaleimide (NEM) treatment and can be restored by the addition of NEM-sensitive factor (NSF) (Diaz *et al*., 1989). To determine the role of NSF in early endosome fusion in *Leishmania*, fusion was carried out in the presence of different concentrations of NEM. Figure 6A shows that ~70% of the fusion was inhibited by 0.3 mM NEM. Endosome fusion was also significantly abrogated in cytosol immunodepleted with antibody against mammalian NSF, which detected an ~70 kDa protein, presumably the NSF homolog, in *Leishmania* lysate (Figure 6B, inset).

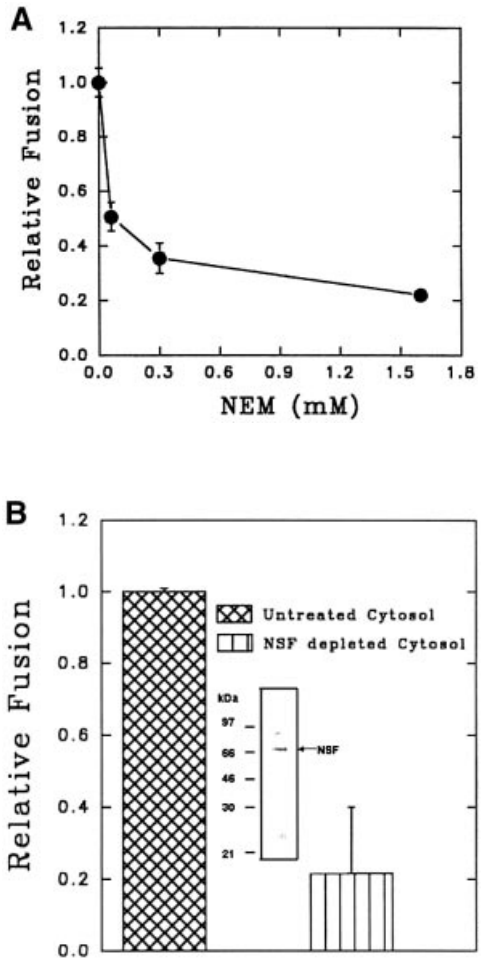

Fig. 6. Role of NSF-like protein in endosome fusion in *Leishmania*. (**A**) Cytosol prepared from *Leishmania* was treated with different concentrations of NEM (30 min at 4°C) and excess NEM was quenched with 3 mM DTT. Fusion was carried out in an ATP-regenerating system containing NEM-treated cytosol. Fusion obtained in the absence of NEM was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 10.6 ng of HRP activity/mg of protein. (**B**) Endosome fusion was carried out in the presence of NSF-immunodepleted cytosol as indicated in Materials and methods. Fusion obtained with untreated cytosol was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 11.2 ng of HRP activity/mg of protein. Inset shows western blot analysis of *Leishmania* cell lysate (40 μg/ lane) with antibodies against mammalian NSF. Results from western blots are representative of three independent preparations.

Role of endocytic Rabs in intracellular trafficking of Hb

To determine the role of endocytic Rabs in endosome fusion in *Leishmania*, fusion of BHb-loaded early endosomes with early endosomes containing AHRP was carried out in the presence of Rab5- or Rab7-immunodepleted cytosol. Anti-LdRab5 and anti-LdRab7 specifically depleted the respective proteins from *Leishmania* cytosol (Figure 7A, inset). Fusion between early endosomes was inhibited by ~75% in Rab5-depleted cytosol, whereas Rab7 depletion reduced fusion by only ~25% (Figure 7A). Addition of *in vitro* prenylated LdRab5 (Lombardi *et al*., 1993) to the Rab5-depleted system completely restored fusion to the control level (Figure 7A). Previous studies have shown that early endosomes can fuse with early endosomes, whereas *in vitro* fusion between early and late endosomes does not occur (Gorvel *et al*., 1991). This prompted us to test heterotypic fusion between early and late endosomes in *Leishmania*. Accordingly, late endosomal fractions were prepared by 5 min internalization of AHRP followed by 15 min chase at 23°C, as described previously (Gorvel *et al*., 1991; Laurent *et al*., 1998), and separated by sucrose gradient. Partial characterization of fractions containing maximum HRP activity revealed that these vesicles predominantly contain Rab7, a late endosomal marker, but no Rab5 or transferrin receptor, whereas 5 min internalized AHRP vesicles possess early endosomal markers such as Rab5 and transferrin receptor (Figure 7B). In contrast to the previous study (Gorvel *et al*., 1991), ~80% fusion between BHb-loaded early endosomes and late endosomes containing AHRP was observed in *Leishmania* (Figure 7C). However, consistent with the earlier report, Figure 7C shows that early endosomes containing fluid phase markers such as avidin and biotinylated HRP (BHRP) fuse efficiently, whereas fusions of early endosomes containing avidin with late endosomes containing BHRP is significantly inhibited. Moreover, Figure 7D shows that the observed heterotypic fusion between BHb-loaded early endosomes and late endosome containing AHRP is significantly inhibited in the presence of Rab7-depleted cytosol but not by Rab5-depleted cytosol. Addition of *in vitro* prenylated LdRab7 to the Rab7-depleted system significantly restored fusion (Figure 7D). These results indicate that the cytoplasmic tail of the Hb receptor (HbR) from early endosomes containing BHb may possibly promote this heterotypic fusion.

To determine the role of the cytoplasmic tail of the HbR in heterotypic fusion, we have cloned and expressed different deletion mutants of the receptor as GST fusion proteins (Figure 8A): the N-terminus (HbR-ΔC), middle region (HbR-ΔNC) and C-terminus (HbR-ΔN). Figure 8B shows that polyclonal antibody against HbR purified from *Leishmania* (PHbR) recognized HbR-ΔNC and HbR-ΔN, while a monoclonal antibody (1B6) specifically recognized HbR-ΔN. In addition, HbR-ΔC predominantly bound to Hb, in comparison with other fragments. PHbR was used as a positive control. No binding was observed with GST (data not shown). When BHb-loaded early endosomes were pre-treated with 1B6, heterotypic fusion with late endosomes containing AHRP was significantly inhibited. Moreover, addition of HbR-ΔN in the fusion assay inhibited ~80% of fusion, whereas no significant inhibition was observed with HbR-ΔC or HbR-ΔNC (Figure 8C).

Localization of Rab5 in Leishmania

Immunolocalization showed that *L.donovani* Rab5 is localized in the anterior end near the flagellar reservoir (Figure 9G), which is indicated by the presence of nucleus and kinetoplast (Figure 9D). Also, Rab5 co-localized with 5 min internalized Hb (Figure 9J), which is clearly separated from the Lysotracker Red-labeled perinuclear lysosomal compartment (Figure 9M).

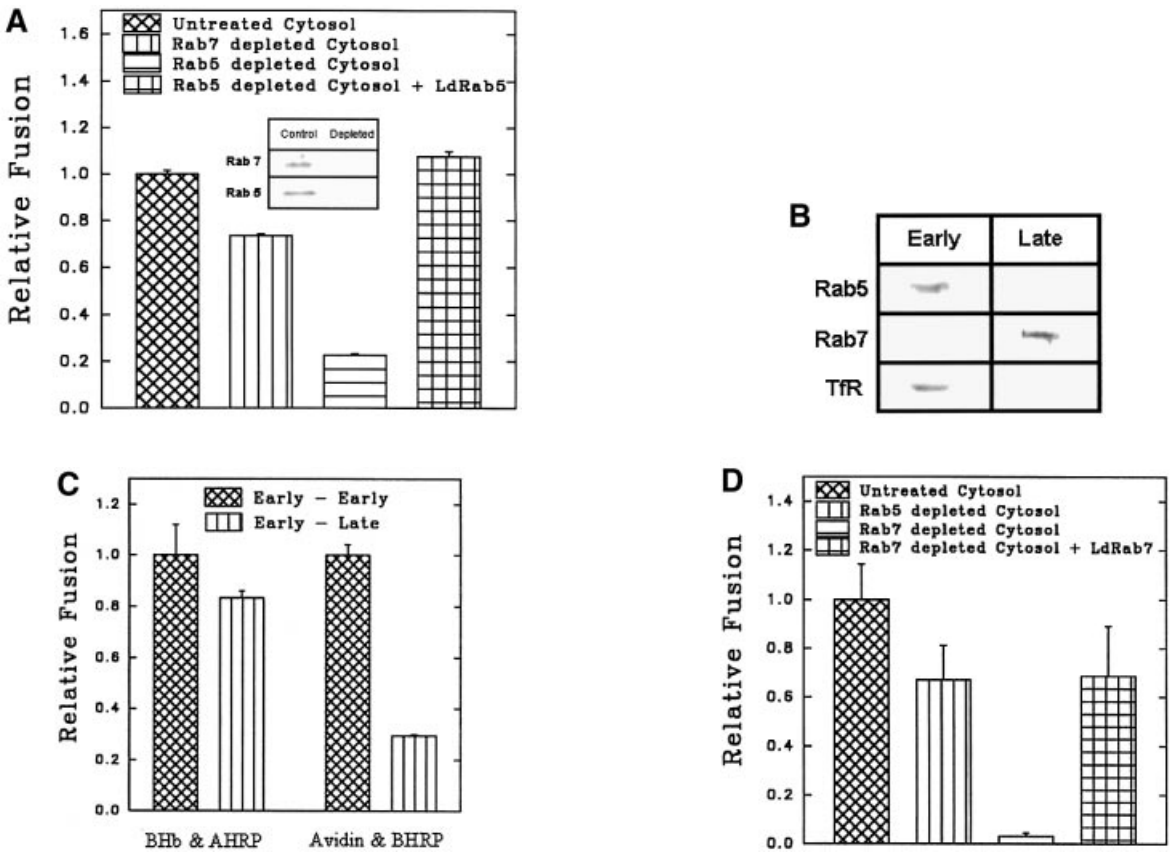

Fig. 7. Role of endocytic Rabs in endosome fusion in *Leishmania*. (**A**) Fusion between early endosomes containing BHb or AHRP was carried out in an ATP-regenerating system in the presence of untreated cytosol, LdRab7-depleted cytosol, LdRab5-depleted cytosol or LdRab5-depleted cytosol supplemented with 500 ng of *in vitro* prenylated LdRab5 as described in Materials and methods. Fusion obtained with untreated cytosol was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 13.2 ng of HRP activity/mg of protein. Inset shows immunodepletion of Rab5 or Rab7 from *Leishmania* cytosol using specific antibodies. (**B**) AHRP-labeled early and late endosomes purified by sucrose density gradient were analyzed for the presence of early and late compartment-specific markers by western blot using specific antibodies (TfR, transferrin receptor). (**C**) Fusion of early endosomes containing BHb with early or late endosomes containing AHRP was carried out in an ATP-regenerating system in the presence of cytosol. Similarly, fusion of early endosomes containing fluid phase marker such as avidin was carried out with early or late endosomes containing BHRP. Fusion obtained between the early endosomes in the respective system was chosen as 1 U, and results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 15.2 and 12.8 ng of HRP activity/mg of protein in the fusion assay of early endosomes containing BHb and avidin, respectively. (**D**) Heterotypic fusion between BHb-loaded early endosomes and late endosomes containing AHRP was carried out in an ATP-regenerating system in the presence of untreated cytosol, LdRab5-depleted cytosol, LdRab7-depleted cytosol or LdRab7-depleted cytosol supplemented with 500 ng of *in vitro* prenylated LdRab7. Fusion obtained with untreated cytosol was chosen as 1 U, and results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 12.4 ng of HRP activity/mg of protein.

Discussion

To understand the intracellular trafficking of Hb in *L.donovani* promastigotes and its sorting in the early endocytic compartment, we have used an *in vitro* reconstitution assay (Gruenberg *et al*., 1989) of endosomes isolated from *Leishmania* promastigotes using appropriate receptor-mediated or fluid phase endocytic probes. The cell fractionation data and partial characterization of purified vesicles revealed that 5 min internalization of an appropriate endocytic probe specifically labeled early endosomes enriched in Rab5 and transferrin receptor, whereas 5 min internalization followed by 15 min chase specifically labeled late endosomes containing Rab7, as observed in previous studies (Gorvel *et al*., 1991; Laurent *et al*., 1998). These results are in agreement with the findings that Rab5-positive early endosomes regulate early events of endocytosis (Gorvel *et al*., 1991; Mukhopadhyay *et al*., 1997a), whereas Rab7, localized in late endosomes, serves as a targeting signal to the late compartment (Feng *et al*., 1995).

Our results show that several features of homotypic early endosome fusion in *Leishmania* are similar to fusion events described previously in mammalian cells (Gruenberg *et al*., 1989; Gorvel *et al*., 1991). Thus, early endosome fusion in *Leishmania* requires cytosol, ATP and its hydrolysis. A significantly higher level of fusion between endosomes from *Leishmania* was observed at its optimal growth temperature of 23°C than at 37°C (data not shown). This is not surprising since *Dictyostelium*, which grows optimally at 21–28°C, exhibits significant fusion at a similar temperature (Lenhard *et al*., 1992). It has been shown in several systems that Rab-GDI in the presence of GDP specifically depletes Rab proteins from the mem-

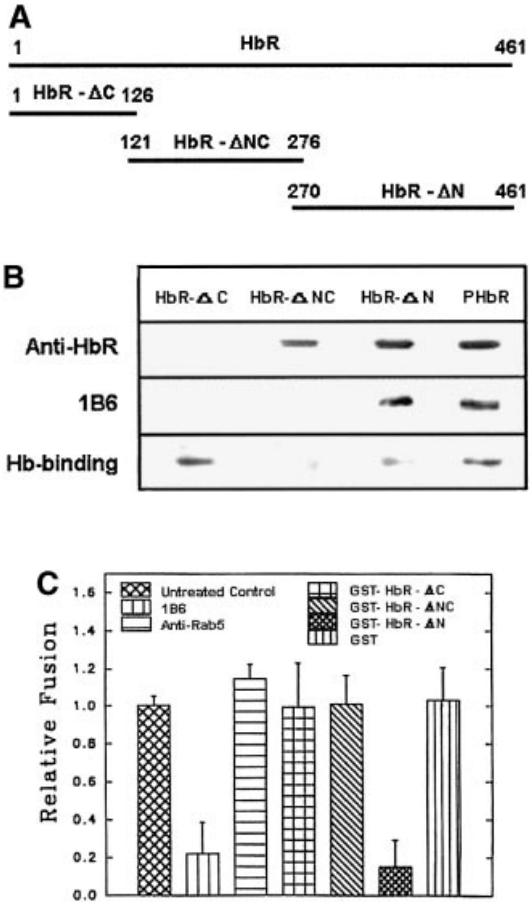

Fig. 8. Role of HbR cytoplasmic tail in heterotypic fusion in *Leishmania.* (**A**) Deletion mutants HbR-ΔC, HbR-ΔNC and HbR-ΔN, corresponding to the N-terminus, middle region and C-terminus of HbR, respectively, were expressed and purified as GST fusion proteins. (**B**) Purified HbR and deletion mutants (1 μg/lane) were analyzed by western blot using anti-HbR polyclonal antibody (top), monoclonal antibody, 1B6 (middle) and hemoglobin binding (bottom) as described in Materials and methods. (**C**) To determine the role of the HbR cytoplasmic domain, BHb-containing early endosomes were incubated with 1B6, anti-LdRab5 antibody or the indicated deletion mutants of HbR (280 ng) for 30 min at 4°C. Subsequently, late endosomes containing AHRP were added to treated early endosomes along with cytosol containing an ATP-regenerating system, and fusion was carried out as described in Materials and methods. Fusion obtained with untreated endosomes was chosen as 1 U, and the results are expressed as relative fusion of three independent experiments ± SD. One unit corresponds to 12.6 ng of HRP activity/mg of protein.

brane (Funato *et al*., 1997) and exhibits broad substrate specificity across species (Attal and Langsley, 1996; Chaturvedi *et al*., 1999). In agreement with this, mammalian GDI along with GDP selectively stripped off *Leishmania* Rab protein from the endosomes, rendering them fusion incompetent, demonstrating the role of Rab proteins in this fusion.

Another ubiquitous factor required for vesicle fusion in mammalian cells is NSF, a homohexamer having both ATP-binding and hydrolyzing activities (May *et al*., 2001). The current model suggests that NSF in its ATP-bound state binds to the membrane through soluble NSF attachment protein (SNAP), and ATP hydrolysis of NSF triggers rearrangement of v-SNARE and t-SNARE (SNAP receptor), which actually mediate membrane fusion (Chen and Scheller, 2001). As NSF is an ATPase and because our findings that ATPγS and NEM treatment inhibit fusion of *Leishmania* endosomes, we explored the role of NSF-like protein in this fusion. NSF is reported to be well conserved among different organisms, and antibodies against NSF from one organism cross-react with others (Weidenhaupt *et al*., 1998). Accordingly, in our study, anti-mammalian NSF antibody specifically recognizes an ~70 kDa protein in *Leishmania*, and fusion carried out in the presence of cytosol immunodepleted using this antibody is significantly inhibited, demonstrating the role of NSF-like protein in endosome fusion in *Leishmania*. These results along with others suggest that, as in higher eukaryotic cells, an NSF-mediated SNARE complex is likely to regulate endocytosis in unicellular protozoa (Chaturvedi *et al*., 1999; Bogdanovic *et al*., 2000).

Previous studies have shown that Rab5 regulates homotypic fusion between early endosomes. To determine the role of Rab5 in endosome fusion in *Leishmania*, we have cloned and expressed LdRab5, which specifically binds GTP. However, GTPase activity of LdRab5 is lower than mammalian Rab5. It has been shown that consensus sequences of Rabs in switch I (IGVDF) and switch II (KLQIW) regions are crucial for GTP hydrolysis and GDP/GTP exchange and this sequence is sensitive to alteration. The switch I and switch II regions of LdRab5 consist of VGASF and HFDIW, respectively, which may possibly explain the relatively low GTPase activity of LdRab5 as compared with its mammalian equivalent. However, LdRab5 contains RYKS and YYRGA, the signature motifs of the Rab subfamily (Pereira-Leal and Seabra, 2000; Stenmark and Olkkonen, 2001). Immunolocalization shows that Rab5 co-localizes with the 5 min internalized Hb-containing compartment, indicating that Rab5 in *Leishmania* is localized in an early endocytic compartment. Recently, it has been shown that LmRab7 localized in the perinuclear late endosome/lysosome compartment in *L.mexicana* (Denny *et al*., 2002). Subsequently, LdRab5, LdRab7 and specific antibodies were used to characterize endosome fusion in *Leishmania*. We observe that LdRab5 regulates fusion of BHb-loaded early endosomes with early endosomes containing AHRP from *Leishmania*, resembling the results of an earlier report (Gorvel *et al*., 1991). However, early endosome fusion in *Dictyostelium* appears to be regulated by Rab7, suggesting that Rab7 may couple both ends of the endocytic pathway in *D.discoideum* (Laurent *et al*., 1998).

It has been shown in different reconstitution systems that early endosomes are capable of homotypic fusion *in vitro*, whereas heterotypic fusion between early endosomes and late endosomes does not occur *in vitro* (Gorvel *et al*., 1991; Laurent *et al*., 1998). Similarly, early and late endosomes prepared from *Leishmania* promastigotes after fluid phase uptake of avidin and BHRP, respectively, do not fuse as observed in other systems. In contrast, early endosomes containing a receptor-mediated endocytic probe, BHb, drive the fusion with both early and late compartments in *Leishmania*, and Rab7 regulates this heterotypic fusion.

Several studies indicate that the signals for endocytosis and intracellular trafficking often reside in the cytoplasmic domain of the receptor. For example, deletion of core kinase sequences from the distal region of the cytoplasmic

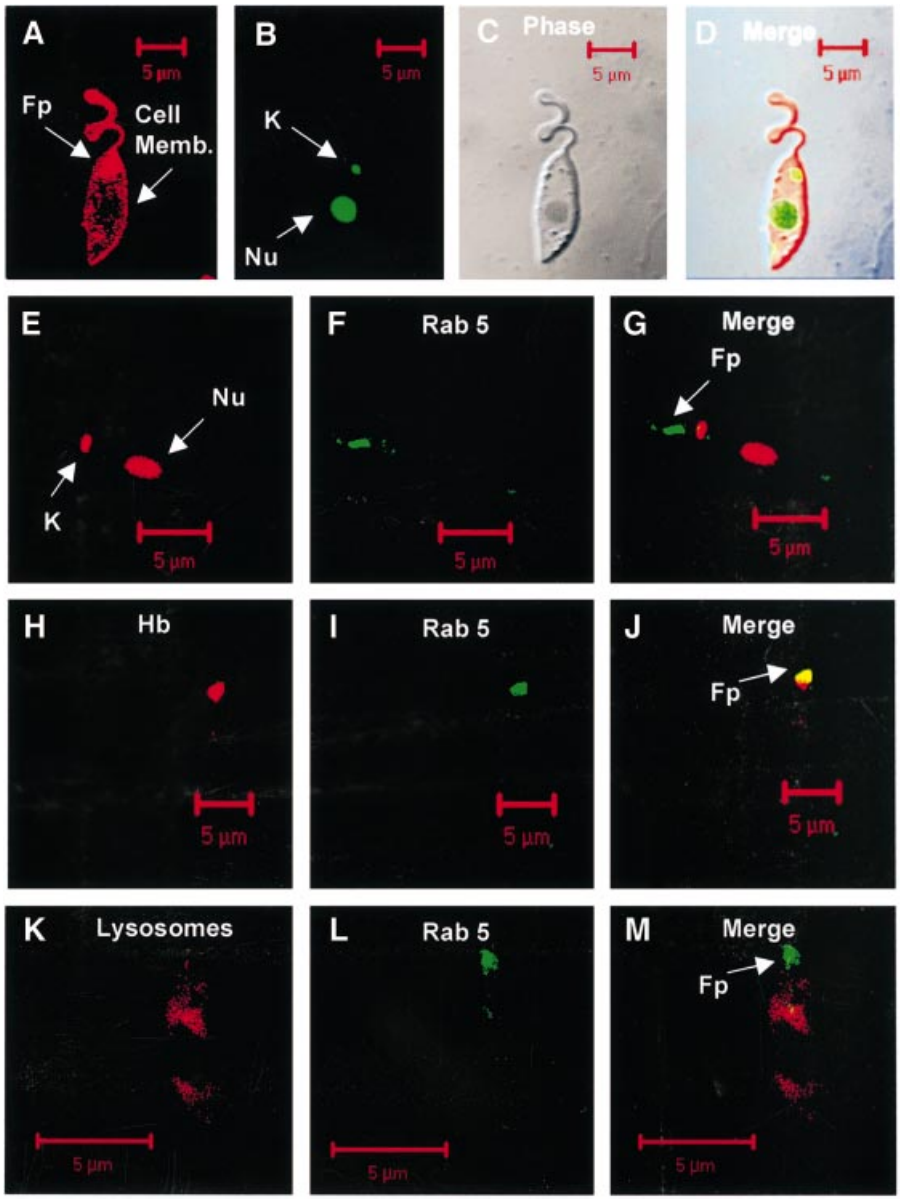

Fig. 9. Confocal images showing the localization of Rab5 in *L.donovani* promastigotes. Cells were fixed in formaldehyde and Rab5 was visualized by anti-LdRab5 antibody and subsequently probed with goat anti-mouse Alexa Fluor 488-labeled second antibody (**F**, **G**, **I**, **J**, **L** and **M**) as described in Materials and methods. Early endosomes were visualized by 5 min uptake of BHb, probed with avidin–Texas red (**H** and J). Yellow indicates co-localization of Rab5 with Hb in early endosomes (J). Nucleus (Nu) and kinetoplast (K) were labeled with propidium iodide (**E** and G), and lysosome-like compartments were visualized by pre-incubating the promastigotes with Lysotracker Red (**K** and M) for 30 min at 23°C. The upper panel shows the phase image (**C**) of plasma membrane labeled with FM4-64 (**A**) and kinetoplast and nucleus stained with Syto green (**B** and **D**). Fp indicates the flagellar pocket.

domain of the epidermal growth factor (EGF) receptor impaired proper trafficking of the receptor to the late/lysosomal compartment (Kornilova *et al*., 1996). Similarly, a sequence distal to the endocytic motif of cation-independent mannose 6-phosphate receptor in the cytoplasmic tail is required for efficient transport to late endosomes (Juuti-Uusitalo *et al*., 2000). In addition, dendritic cells express DEC-205, an endocytic receptor-like macrophage mannose receptor (MMR). However, unlike MMR, DEC-205 receptor recycles from the late compartment and the targeting signal is localized in the distal region of the cytoplasmic tail (Mahnke *et al*., 2000). Thus, it is tempting to speculate that the cytoplasmic tail of HbR projecting from the early endosome may transduce some signal(s) to mediate fusion with late endosomes in *Leishmania*.

In order to prove unequivocally that the cytoplasmic domain of the HbR regulates heterotypic fusion, we have cloned and expressed different deletion mutants of HbR, which is a transmembrane protein having kinase activity (data not shown). Topology prediction (TMPred; Hofmann and Stoffel, 1993) of HbR sequence and the observed maximum binding of Hb with HbR-ΔC suggest that possibly the N-terminus is the extracellular domain of

HbR. Our results show that BHb-loaded early endosomes pre-treated with monoclonal antibody (1B6), specific to the C-terminus of HbR, significantly inhibit heterotypic fusion with late endosomes containing AHRP. Similarly, addition of HbR-ΔN in the fusion assay inhibited ~80% of fusion, whereas no significant inhibition was observed with HbR-ΔC or HbR-ΔNC. These results demonstrate that signal transduced from the HbR tail projecting from the early endosomal compartment is blocked by a C-terminus-specific antibody or competed by HbR-ΔN, indicating that the signal mediated through the C-terminal cytoplasmic tail of HbR may promote the fusion with late endosomes.

In conclusion, our results represent the first documentation that endocytosis in unicellular parasitic protozoa such as *Leishmania* is regulated by small GTP-binding proteins of the Rab family through vesicle fusion. Interestingly, our results have shown that early endosomes containing Hb in *Leishmania* fuse efficiently with both early and late compartments. We suggest that Hb in *Leishmania* first moves to an early endosomal compartment where Rab5-dependent rapid exchange between the endosomes occurs. Subsequently, Hb is targeted to the late/lysosomal compartment through signals mediated by the cytoplasmic tail of the receptor, which is Rab7 dependent. It will be interesting to determine the nature of signal(s) mediated through the receptor tail, which promotes heterotypic fusion between early endosomes and late endosomes in *Leishmania*.

Materials and methods

Materials

Avidin, AHRP, *N*-hydroxy succicinimidobiotin (NHS-Bio) and bicinchoninic acid (BCA) were purchased from Pierce Biochemicals (Rockford, IL). Recombinant mammalian GDI and Rab5 constructs were kindly provided by Dr Philip Stahl (Washington University School of Medicine, St Louis, MO). A mouse monoclonal anti-Rab5 antibody was received as a gift from Dr A.Wandinger-Ness (Northwestern University, Evanston, IL). Alexa Fluor 488, goat anti-mouse IgG and Lysotracker Red were purchased from Molecular Probes (Eugene, OR). All HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Amersham, UK). Unless otherwise stated, all other reagents were obtained from Sigma Chemical Co (St. Louis, MO). Anti-Hb, anti-HbR and anti-NSF polyclonal or monoclonal antibodies were raised in mice by standard techniques (Overkamp *et al*., 1988; Celis *et al*., 1994). Hb was biotinylated using NHS-Bio as described previously (Gruenberg *et al*., 1989). Cytosol was prepared from *Leishmania* using a procedure similar to that described previously (Mukherjee *et al*., 2000).

Cells

Leishmania donovani promastigotes (UR 6) were obtained from the Indian Institute of Chemical Biology, Kolkata, India. Cells were routinely maintained on blood agar slants containing glucose, peptone, sodium chloride, beef heart extract, rabbit blood and gentamycin (Roy, 1932). For experimental use, cells were harvested in phosphate-buffered (10 mM pH 7.2) saline (0.15 M) from 3-day-old slants.

Cloning and expression of Rab5 homolog from L.donovani

A putative Rab5-like sequence with substantial homology to *T.brucei* Rab5B was identified from the *L.major* genome using BLAST. Accordingly, forward (5′-GGATCCATGTCATCCATCAGTCGC-3′) and reverse (5′-GAATTCCTAGCAGCATCCGTTCTCT-3′) primers were designed against the start and stop codons, respectively, of the putative Rab5 sequence of *L.major*. RT–PCR was performed using these primers to amplify the open reading frame of the putative Rab5 sequence using *L.donovani* cDNA. mRNA isolated from *Leishmania* promastigotes using an Oligotex mRNA kit (Qiagen, Germany) was used for cDNA synthesis using a Thermo Script RT–PCR kit (Gibco-BRL) as per the manufacturer's instructions. Subsequently, PCR was performed using the above primers in a Perkin-Elmer thermocycler for 30 cycles: denaturation at 94°C for 3 min; annealing at 55°C for 30 s; and extension at 72°C for 1 min. The PCR product was cloned into pGEMT-easy vector (Promega) and sequenced using m13 universal primers in an automated sequencer. Subsequently, the PCR product was cloned into *Bam*HI–*Eco*RI sites of the pGEX-4T-2 vector (Amersham Biosciences) and transformed into *Escherichia coli*.

Expression and purification of LdRab5

Transformed *E.coli* were grown in LB and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C for expression of GST–LdRab5. Cells were harvested, lysed by sonication and lysate was treated with Triton X-100 (1%). Cellular debris was removed by centrifugation at 13 000 *g* for 10 min at 4°C. The fusion protein, GST–LdRab5, was purified from the supernatant using reduced glutathione beads by standard procedures.

Generation of antibodies against LdRab5 and LdRab7

GST–LdRab5 immobilized on glutathione beads was cleaved with 10 U of thrombin (Pharmacia) in cleavage buffer (50 mM Tris–HCl pH 7.5 containing 150 mM NaCl and 2.5 mM $CaCl_2$) for 3 h at 24°C, and beads were removed by centrifugation (Smith and Corcoran, 1993). After ensuring the purity of the released LdRab5 by SDS–PAGE, mice were immunized with this protein to raise antibodies by standard techniques (Overkamp *et al*., 1988). We have also cloned and expressed Rab7 from *L.donovani* (data not shown) and raised antibody against LdRab7 using a similar procedure. The specificity of the antibodies against the respective proteins was determined by western blot analysis using *Leishmania* cell lysate.

GTP overlay assay

GTP binding of LdRab5 was detected in an overlay assay (Via *et al*., 1997). Briefly, different amounts of GST–LdRab5 and mammalian GST–Rab5 proteins blotted onto nitrocellulose membranes were incubated with 1 μCi/ml of [α-^{32}P]GTP (3000 Ci/mM, NEN) in 50 mM phosphate buffer pH 7.5 containing 5 mM $MgCl_2$, 1 mM EGTA and 0.3% Tween-20, washed and visualized by autoradiography.

GTPase assay

The GTPase activity of LdRab5 was determined as described previously (Stenmark *et al*., 1994). Briefly, 2 pmol of immobilized Rab5 was incubated with buffer A (20 mM Tris–HCl pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM Na-phosphate and 10 mM 2-mercaptoethanol) for 20 min at 25°C and bound nucleotides were eluted with 1 M guanidine-HCl. Immobilized proteins were then incubated with 2 pmol [α-^{32}P]GTP (800 Ci/mmol, Perkin Elmer Life Sciences, MA) in 20 μl of buffer A for 10 min at 0°C and incubated for various times at 23°C. Samples were washed and incubated in 8 μl of buffer B (0.2% SDS, 2 mM EDTA, 10 mM GDP, 10 mM GTP pH 7.5) and heated for 2 min at 70°C. An aliquot was analyzed using thin-layer chromatography and visualized by autoradiography.

Preparation of early endosomes from Leishmania

Leishmania promastigotes were incubated with BHb (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. Internalization of BHb was stopped by adding cold medium, and 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). Uninternalized BHb bound to the cells was quenched by incubating the cells with excess avidin (200 μg/ml) at 4°C for 30 min. After washing with HB, 5×10^9 cells were resuspended in 12 ml of HB, equilibrated in a pre-cooled nitrogen cavitation bomb (Parr Instrument company, IL) with 750 p.s.i. N_2 for 25 min and disrupted by release of N_2 from the bomb (Shapiro *et al*., 1989). The unbroken cells, nuclei and other cell debris were removed by low speed centrifugation at 500 *g* for 10 min at 4°C. The post-nuclear supernatant (PNS) was snap-frozen in liquid nitrogen. The enriched endosomal fraction from the PNS was prepared as described previously (Mukherjee *et al*., 2000). Briefly, thawed PNS was diluted with HB (1:3) and centrifuged at 20 000 *g* for 1 min at 4°C. The resultant supernatant was again centrifuged at 100 000 *g* for 5 min at 4°C. The pellet enriched in early endosomal vesicles was used for *in vitro* fusion assays. Another set of endosomes containing AHRP was prepared similarly.

Likewise, late endosomes were labeled by 5 min internalization followed by 15 min chase of AHRP or BHRP at 23°C. HRP-labeled enriched late endosomal vesicles were purified from the sucrose gradient as described below.

Subcellular fractionation of L.donovani endocytic compartment

To characterize the compartments labeled by 5 min internalization of AHRP by *L.donovani* promastigotes at 23°C, subcellular fractionation was carried out as described previously (Laurent *et al*., 1998). Briefly, cells were disrupted after internalization of AHRP and 0.5 ml of PNS 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 *g* for 1 h at 4°C, 50 µl fractions were collected from the top of the gradient and samples were analyzed for the presence of HRP, 5′-nucleotidase activity (Graham, 1993) and various compartment-specific markers. A similar fractionation was carried out using BHb as probe, and the presence of Hb in fractions was detected by western blot analysis using anti-Hb antibody.

In vitro reconstitution of endosome fusion in Leishmania

Reconstitution of fusion between endosomes prepared from *Leishmania* was carried out as described earlier (Gorvel *et al*., 1991). Briefly, two sets of endosomes containing BHb or AHRP were mixed in fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH pH 7.2, 1 mM DTT, 1.5 mM $MgCl_2$, 100 mM KCl, including an ATP-regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 U/ml creatine phosphokinase and 0.25 mg/ml avidin as scavenger) supplemented with gel-filtered (G-25 Sephadex) cytosol prepared from *Leishmania.* Fusion was carried out for 1 h at 23°C and the reaction was stopped by chilling on ice. The membrane was solubilized in solubilization buffer (SB; PBS containing 1% Triton X-100 and 0.2% methylbenzethonium chloride with 0.25 mg/ml avidin as scavenger), and BHb–AHRP complexes were immunoprecipitated using anti-Hb antibody. The HRP activity associated with the BHb–AHRP complex was measured as fusion units using *O*-phenylenediamine as chromogenic substrate (Gruenberg *et al*., 1989). Fusion carried out in the absence of cytosol was low and was subtracted from the corresponding values to determine specific fusion. Maximum fusion obtained at 1 mg/ml cytosol concentration was expressed as 1 U of relative fusion. HRP activity corresponding to 1 U is specified in the figure legends.

Immunodepletion of Leishmania cytosol using specific antibodies

To deplete Rab5 from *Leishmania* cytosol, 100 µl of protein A/G plus–agarose (Santa Cruz Biotechnology) was incubated with 10 µl of anti-LdRab5 antibody in PBS overnight at 4°C. The antibody–protein A/G–agarose complex was washed and blocked with 1 mg/ml bovine serum albumin (BSA) for 1 h at 4°C. Subsequently, 600 µg of cytosol containing protease inhibitors was added to the protein A/G–agarose–anti-LdRab5 complex and incubated for 1 h at 4°C as described previously (Mukherjee *et al*., 2001). Rab5-depleted *Leishmania* cytosol was separated from the agarose beads by centrifugation. NSF- or Rab7-depleted *Leishmania* cytosol was prepared similarly using the respective antibodies.

Generation of HbR deletion mutants

Recently we have cloned and expressed HbR from *L.donovani* (data not shown). Different deletion mutants corresponding to the 5′ end (1–378 bp), middle region (363–828 bp) and 3′ end (810–1383 bp) of the gene were amplified by PCR using appropriate forward and reserve primers designed against the HbR sequence. These fragments were cloned into pGEMT-easy vector, sequenced and subcloned into pGEX-4T-2 vector. The GST fusion proteins, HbR-ΔC, HbR-ΔNC and HbR-ΔN, corresponding to the N-terminus, middle region and C-terminus of HbR, respectively, were expressed in *E.coli* and purified by standard procedure.

Hemoglobin binding activity of HbR

Purified HbR and different deletion mutants were subjected to SDS–PAGE (1 µg of each protein) and transferred to a nitrocellulose membrane. After blocking with 5% BSA and incubating with 1 µg/ml Hb, the membrane was incubated with anti-Hb antibody and subsequently probed with HRP-labeled secondary antibodies. Hb bound to various proteins was visualized by ECL.

Immunolocalization of Rab5 in L.donovani

In order to localize Rab5 in *L.donovani* promastigotes, cells were washed three times with PBS and fixed in 4% formaldehyde in PBS for 20 min at 4°C. Following the washes, thin smears of cells were prepared on glass slides and permeabilized with 0.4% saponin for 20 min at 24°C. Subsequently, cells were incubated with 10% fetal calf serum (FCS) to block non-specific binding, washed and probed with anti-LdRab5 antibody in PBS containing 0.4% saponin for 30 min at 24°C. Cells were washed three times with PBS and incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody in the same buffer for 30 min at 24°C. Finally, the cells were washed and incubated for 15 min at 24°C with 5 µg/ml of propidium iodide to label the nucleus and kinetoplast. Similarly, early endosomes were labeled by 2 h binding of BHb at 4°C, washed, followed by 5 min uptake at 23°C and visualized by avidin–Texas red. Promastigotes were pre-incubated with Lysostracker Red (10 µM) for 30 min at 23°C in PBS to label the lysosome-like compartment, before fixing the cells. Slides were mounted with antifade reagents (Molecular probes) and viewed in an LSM 510 confocal microscope using an oil immersion objective.

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