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

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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; Pai 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 Trypanosoma 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

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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 [γ-32P]GTP. The binding of [γ-32P]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
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 MgCl2, 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
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).
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-NC), middle region (HbR-NC) and C-terminus (HbR-NC). Figure 8B shows that polyclonal antibody against HbR purified from *Leishmania* (PHbR) recognized HbR-NC and HbR-CN, while a monoclonal antibody (1B6) specifically recognized HbR-NC. In addition, HbR-NC 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-CN in the fusion assay inhibited ~80% of fusion, whereas no significant inhibition was observed with HbR-NC or HbR-CN (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).
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-

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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 (TIR, 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.
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 ATPβS 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 Rab 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; Stemkorn 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 Dictostelium 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
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 heterotopic fusion between early endosomes and late endosomes in Leishmania.

Materials and methods

Materials
Avidin, AHRP, N-hydroxy succinimimidobiotin (NHS-Bio) and biocinchonic acid (BCA) were purchased from Pierce Biochemicals (Rockford, IL). Recombinant mammalian GDI and Rab5 constructs were kindly provided by Dr Phillip 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 Avidin, AHRP, was prepared similarly.

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 25°C, to label the early endosomal compartment. Internalization of BHb was stopped by adding cold internalization medium (MEM containing 10 mM HEPES and 5 mM glucose). Cells were washed and incubated in 8 µl of buffer B (0.2% SDS, 2 mM EDTA, 10 mM MgCl2, 1 mM EGTA, pH 7.2 containing protease inhibitors). Unteinated 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 x 10^6 cells were resuspended in 12 ml of HB, equilibrated in a pre-cooled nitrogen caviation bomb (Parr Instrument company, IL) with 750 p.s.i. N2 for 25 min and disrupted by release of N2 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 \textit{L. donovani} endocytic compartment

To characterize the compartments labeled by 5 min internalization of AHRP by \textit{L. donovani} promastigotes at 23°C, subcellular fractionation was carried out as described previously (Laurent \textit{et al}., 1998). Briefly, cells were disrupted after internalization of AHRP and 0.5 ml of PNS was carried out as described previously (Laurent \textit{et al}., 1998). Promastigotes which were pre-incubated with Lysostrecker Red (10 \textmu M) 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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