Chapter 3

Role of hemoglobin receptor tail in the fusion between early and late endosomes.

5.1 Introduction

Receptor-mediated endocytosis is the mechanism by which a variety of nutrients, growth factors etc. are efficiently transported inside cells (Goldstein *et al.*, 1985). In this process, receptors are selectively concentrated in clathrin coated pits and bind specific ligand. Subsequently, bound ligand is internalized and delivered to early endosomes, peripheral membrane bound compartments, where receptors and their cargo are sorted for recycling to plasma membrane, delivery to other intracellular compartments or transport to lysosomes for degradation (Smythe and Warren, 1991). Thus, the first station in the endocytic process is the early endosomes where receptors are sorted into tubular endosomal extension for recycling back to the plasma membrane and cargo to be degraded translocate towards the cell center into more spherical multivesicular compartments known as late endosomes (Mellman, 1996), the precursors of lysosomes. It has been shown that transport from early to late endosomes is dependent on microtubule filaments (Gruenberg and Howell, 1989). Concomitant with each of these events, early endosomes fuse with other early endosomes and late endosomes fuse with other late endosomes, by a process referred to as 'homotypic fusion'. Our results have demonstrated that Rab5 GTPase regulates homotypic fusion between early endosomes in *Leishmania,* which is in agreement with previous studies in other systems.

Recent studies also show that Rab5 stimulates both the association of early endosomes with microtubules as well as the movement of early endosomes towards late endosomes (Nielsen *et al.*, 1999). Similarly, early carrier vesicles (ECV), which pinch off from early endosomes, are capable of fusing with the late endosomes *in vitro* but only in the presence of taxol-polymerized microtubules (Aniento *et al.*, 1993). Thus, an intact microtubule system is required for the delivery of cargo from early to late endosomes and lysosomes and depolymerization of microtubules reduces the delivery of internalized molecules to lysosomes (Vale, 1987).

Heterotypic fusion, fusion between two temporally dissimilar vesicles, is known to occur only between the early endosomes and late endosomes in the perfused rat liver (Schmid *et al.*, 1998). However, in cultured cells like BHK and CHO, it has been shown that early endosomes do not fuse efficiently with late endosomes *in vitro* (Aniento *et al.*, 1993; Braell, 1987). Though heterotypic fusion does not occur between endocytic vesicles, reconstitution of transport events between successive

compartments of the Golgi (Balch *et al.*, 1984; Happe *et al.*, 1998), between ER and Golgi (Balch *et al.*, 1987) and between TGN and plasma membrane (Woodman *et al.*, 1986) have been successfully carried out using cultured cells of mammalian origin.

Studies on *in vitro* endosome fusion in *D. discoidium* show that post nuclear supernatant labeled with 5 mins internalized probe does not fuse with post nuclear supernatant containing late compartments, suggesting that heterotypic fusion in *Dictyostelium* does not occur *in vitro,* similar to the mammalian systems (Laurent *et al.*, 1998). We have determined the ability of early endosomes to undergo fusion with the late endosome *in vitro*. We show that early endosomes containing hemoglobin from *Leishmania* are capable of fusing with late endosomes *in vitro* and this process is regulated by signals mediated from hemoglobin receptor tail.

5.2 Materials and Methods

5.2.1 Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co (St. Louis, MO). N-hydroxy succinimidobiotin (NHS-Biotin), avidin-horseradish peroxidase (AHRP) and avidin were purchased from Vector laboratories, Inc (Burlingame CA). Bicinchoninic acid (BCA) reagent was purchased from Pierce Biochemicals, Rockford, IL. Anti-hemoglobin, anti-LdRab7 and anti-LdRab5 antibodies were raised in mice by standard techniques (Overkamp *et al.*, 1988). Monoclonal anti-hemoglobin receptor antibody was raised as described (Celis *et al.*, 1994). Other reagents used were of analytical grade. Protein A/G agarose beads were purchased from Santa Cruz.

5.2.2 Preparation of late endosomes from *Leishmania*

Early endosomes containing AHRP and endosomes containing BHb were prepared as described in chapter 2. To prepare the late endosomes from *Leishmania,* promastigotes were incubated with AHRP (2mg/ml) in internalization medium (MEM, containing 10 mM HEPES and 5mM glucose, pH 7.4) for 5 min at 23°C to label the early endosomal compartment. Internalization of AHRP was stopped by the addition of cold medium and the cells were washed five times with phosphate buffered saline (PBS). Subsequently, cells were incubated in internalization medium for 15 min at 23°C to further chase the probe to the later compartments. Finally, cells

were resuspended (5 x 10^9 cells/12 ml) in homogenization buffer (HB, 20 mM Hepes, 250 mM sucrose and 2 mM EGTA, pH 7.2 containing protease inhibitors). The cell suspension was equilibrated in a pre-cooled nitrogen cavitation vessel (Parr Instrument company, IL) under 750 psi N_2 for 25 min. Cells were disrupted by release of N_2 from the vessel (Shapira *et al.*, 1989). The unbroken cells, nucleus 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 quickly frozen in liquid nitrogen. PNS containing late endosomes labeled with AHRP was used in subsequent experiments. Late endosomes containing biotinylated-HRP were prepared under identical conditions.

5.2.3 *In vitro* reconstitution of heterotypic endosome fusion in *Leishmania*

In order to determine the heterotypic fusion between the early endosomes and late endosomes from *Leishmania*, reconstitution of fusion was carried out using similar procedure described earlier for other systems (Gorvel *et al.*, 1991). Briefly, two sets of endosomes, the early endosomes containing BHb were mixed with late endosomes containing AHRP in fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2, 1 mM dithiothreitol, 1.5 mM $MgCl_2$, 100 mM KCl, including an ATP regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 units/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 hr 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 complex were immunoprecipitated using anti-Hb antibody. The HRP activity associated with BHb-AHRP complex was measured as fusion unit using O-phenylenediamine as the chromogenic substrate (Gruenberg *et al.*, 1989). Similarly, heterotypic fusion between early and late endosomes containing fluid phase markers like avidin and Biotin-HRP, respectively, was carried out under the conditions described above.

5.2.4 Immunodepletion of Rab5 and Rab7 from *Leishmania* cytosol

To determine the role of Rab proteins in *in vitro* heterotypic fusion, *Leishmania* cytosol was depleted of Rab5 or Rab7 and analyzed for depletion by Western blotting using relevant antibodies. Subsequently, fusion between early endosomes containing BHb and late endosomes containing AHRP was carried out in ATP regenerating system in the presence of untreated cytosol, Rab5 depleted cytosol, Rab7 depleted cytosol or Rab7 depleted cytosol supplemented with 500 ng of *in vitro* prenylated LdRab7 as described in the preceding chapter.

5.2.5 Treatment of hemoglobin containing early endosomes with anti-hemoglobin receptor antibody

The early endosomes containing BHb were treated prior to fusion with anti-receptor polyclonal or monoclonal antibody for 30 mins on ice to block the cytoplasmic domain of the receptor. Subsequently, these pretreated early endosomes containing hemoglobin were used in the fusion assay to determine the role of cytoplasmic tail of the receptor in heterotypic fusion.

5.2.6 Generation of hemoglobin receptor deletion mutants

Recently, in a separate study, hemoglobin receptor (HbR) from *L. donovani* was cloned and expressed. Different deletion mutants corresponding to the 5´ end (1-378 bp), middle fragment (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 Hb-receptor sequence. These fragments were cloned into pGEMT-easy vector, sequenced and sub-cloned into pGEX-4T-2 vector. The GST fusion proteins, HbR-ΔC, HbR-ΔNC, HbR-ΔN corresponding to N-terminus, middle region and C-terminus of HbR, respectively, were expressed in *E. coli* and purified by standard procedure.

5.2.7 Hemoglobin binding activity of the hemoglobin receptor and its deletion mutants

Purified HbR and different deletion mutants were subjected to SDS-PAGE (1μg of each protein) and transferred to nitrocellulose membrane. After blocking with 5% BSA, membrane was incubated with 1μg/ ml Hb to allow binding with the

respective receptor fragments. Subsequently, membrane was probed with anti-Hb antibody and binding of primary antibody with the hemoglobin receptor fragment was detected with HRP-labeled secondary antibodies and visualized by ECL.

5.3 Results

5.3.1 Early and Late endosomes in *Leishmania* differ in their biochemical composition

Late endosomes from *Leishmania* 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. Fractions were collected from top of the gradient and analyzed for various markers. Partial characterization of fractions containing maximum HRP activity revealed that these vesicles predominantly contain Rab7, a late endosomal marker, but not Rab5 or transferrin receptor, whereas, 5 min internalized AHRP vesicles possess early endosomal markers like Rab5 and transferrin receptor (Figure 30). Thus, endosomal compartments in *Leishmania* correspond well with the mammalian system in terms of buoyant density, time of chase and the presence of various biochemical makers like Rab5, Rab7 and transferrin receptor.

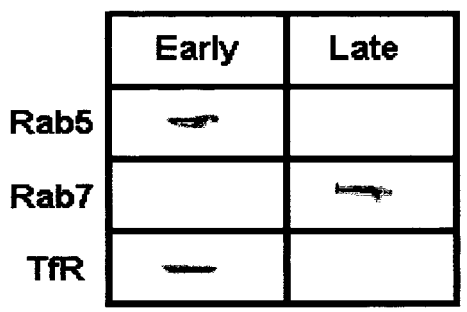

Figure 30. AHRP labeled early and late endosomes purified by sucrose density gradient were analyzed for the presence of early and late compartment-specific markers by Western blot using indicated antibodies (TfR, transferrin receptor).

5.3.2 Comparison of heterotypic fusion between endosomes containing receptor-mediated or fluid phase probes

Previous studies have demonstrated that early endosomes can fuse with each other whereas fusion between early and late endosomes does not occur *in vitro* (Gorvel *et al.*, 1991). Accordingly, the heterotypic fusion between early endosomes

containing BHb and late endosomes containing AHRP was determined in *Leishmania*. In contrast to the previous study (Gorvel *et al.*, 1991), about 80% fusion between BHb-loaded early endosomes and late endosomes containing AHRP was observed in *Leishmania* (Figure 31). To determine whether the observed *in vitro* heterotypic fusion is a general phenomenon in *Leishmania*, early and late endosomes were prepa-

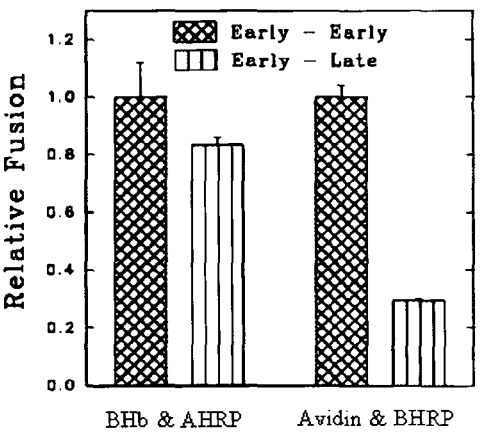

Figure 31. Fusion of early endosomes containing BHb with early or late endosomes containing AHRP were carried out in ATP regenerating system in the presence of cytosol. Similarly, fusion of early endosomes containing fluid phase marker like avidin was carried out with early or late endosomes containing BHRP. Fusion obtained between the early endosomes in the respective system was chosen as 1 unit and results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 15.2 ng and 12.8 ng of HRP activity/mg of protein in the fusion assay of early endosomes containing BHb and avidin,

red using fluid phase markers like avidin and biotin-HRP, respectively. Consistent with previous report, the results presented in the Figure 31 show that early endosomes containing fluid phase markers like avidin and biotin-HRP fuses efficiently whereas fusion of early endosomes containing avidin with late endosomes containing biotin-HRP is significantly inhibited.

5.3.3 Role of Rab proteins in heterotypic fusion.

The observed heterotypic fusion between early endosomes containing BHb and late endosome containing AHRP was significantly inhibited in the presence of

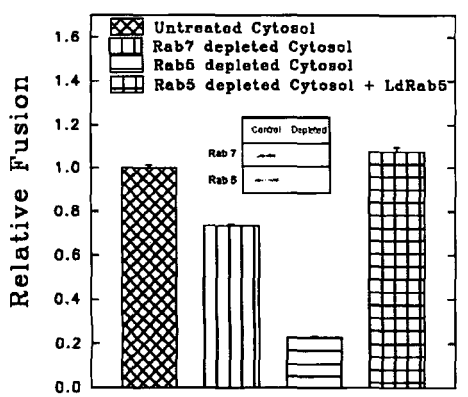

Figure 32: Heterotypic fusion between BHb-loaded early endosomes and late endosomes containing AHRP was carried out in ATP regenerating system in the presence of untreated cytosol, LdRab5 depleted cytosol, LdRab7 depleted cytosol or LdRab7 depleted cytosol supplemented with 500 ng of *in vitro* prenylated LdRab7. Fusion obtained with untreated cytosol was chosen as 1 unit and results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 12.4 ng of HRP activity/mg of protein. The inset shows the western blot of cytosol depleted with specific anti-Rab5 and anti-Rab7 antibodies.

Rab7 depleted cytosol but not by Rab5 depleted cytosol. Furthermore, addition of *in vitro* prenylated recombinant LdRab7 protein to the Rab7 depleted system significantly restored this fusion, demonstrating that heterotypic fusion between these compartments in *Leishmania* is regulated by LdRab7 (Figure 32). These results indicate that the cytoplasmic domain of HbR from early endosomes containing BHb may possibly promote this heterotypic fusion. Depletion of specific Rab protein from the respective cytosol was confirmed by Western blotting (Inset, Figure 32).

5.3.4 Generation of different deletion mutants of hemoglobin receptor and their ability to bind hemoglobin

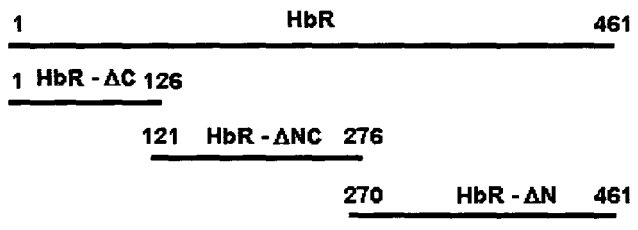

Figure 33. Deletion mutants HbR-ΔC, HbR-ΔNC and HbR-ΔN, corresponding to the N-terminus, middle region and C-terminus of HbR, respectively, were expressed and purified as GST fusion proteins.

Recently, HbR of *Leishmania* was cloned and expressed in our laboratory. To determine the role of cytoplasmic tail of HbR in heterotypic fusion, we have expressed different deletion mutants of the receptor as GST fusion proteins (Figure 33); the N-terminus (HbR-ΔC), middle region (HbR-ΔNC) and C-terminus (HbR-ΔN). Figure 34 shows that the polyclonal antibody raised against HbR purified from *Leishmania* (PHbR) recognized HbR-ΔNC and HbR-ΔN while the monoclonal antibody (1B6), specifically recognized HbR-ΔN. In addition, HbR-ΔC predominantly bound with Hb in comparison to other fragments. Purified hemoglobin receptor (PHbR) was used as positive control. No binding was observed with GST. These results demonstrate that the monoclonal antibody against the hemoglobin receptor

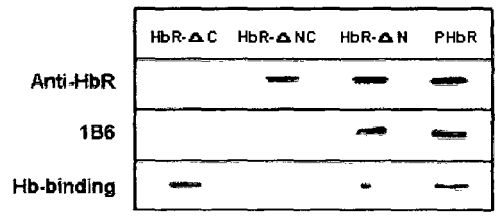

Figure 34. Purified HbR and deletion mutants (1μg/ lane) were analyzed by Western blot using anti-HbR polyclonal antibody (Top), monoclonal antibody, 1B6 (Middle) and their ability to bind hemoglobin (Bottom).

specifically recognizes the C-terminal end of the protein and hemoglobin binding domain of the receptor protein resides in the N-terminus of the protein.

5.3.5 Role of hemoglobin receptor tail in heterotypic fusion

To determine whether the heterotypic fusion between early and late endosomes containing BHb and AHRP, respectively, is regulated by signals mediated through hemoglobin receptor tail, endosomes were pretreated with monoclonal antibody (1B6) specific for C-terminal cytoplasmic domain of the receptor to block any signal generated by the receptor tail. The result presented in the Figure 35 show that heterotypic fusion of BHb containing early endosomes with AHRP containing late endosomes is significantly inhibited when BHb containing early endosomes were pretreated with the monoclonal antibody.

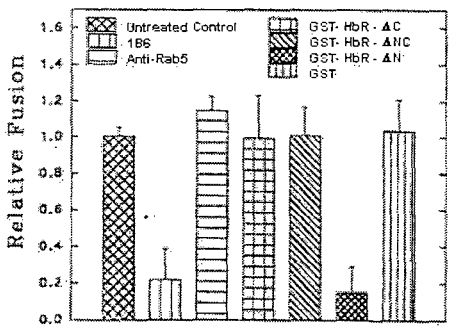

Figure 35. To determine the role of Hb cytoplasmic domain, BHb containing earl endosomes were incubated with 1B6, anti-LdRab antibody or indicated deletion mutants of HbR (28 ng) for 30 min at 4°C. Subsequently, late endosomes containing AHRP were added into treated earl endosomes and fusion was determined. Fusio obtained with untreated endosomes was chosen as unit and the results are expressed as relative fusio of three independent experiments ± S.D. One unit corresponds to 12.6 ng of HRP activity/mg o protein.

In order to unequivocally prove that C-terminal cytoplasmic domain of the receptor projecting from early endosomes containing hemoglobin is involved in regulating heterotypic fusion with late endosomes, fusion was carried out in the presence of different deletion mutants of the hemoglobin receptor. Our results show that addition of HbR-ΔN, a C-terminal cytoplasmic domain specific fragment of hemoglobin receptor, in the fusion assay inhibited about 80% of fusion, whereas no significant inhibition was observed with HbR-ΔC, HbR-ΔNC or free GST (Figure 35). Taken together, these results clearly demonstrate that signals mediated through the cytoplasmic domain of the hemoglobin receptor from the early endosomes promotes fusion with late compartments.

5.4 Discussion

It has been shown in different cell free reconstitution systems that early endosomes are capable of homotypic fusion *in vitro*. In accordance with this, we have observed that homotypic fusion in case of *Leishmania* has several similarities with the mammalian systems (Gruenberg *et al.*, 1989; Barbieri *et al.*, 1994) including the requirement for Rab5 for regulating such homotypic early fusion events. Similar to the endosomal compartments in mammalian cells, the early and late endosomes in *Leishmania* can be differentiated on the basis of buoyant density by sucrose density gradient centrifugation. The early endosomes occur in the lighter fractions whereas the late endosomes are denser. Moreover, compartment specific markers reveal that Rab5 and transferrin receptor are predominantly present in early endosomes whereas the late endosomes are enriched in Rab7 (Figure 30). Thus endosomal compartments in *Leishmania* share similar properties with that of the mammalian cells.

In contrast to homotypic fusion, heterotypic fusion between early endosomes and late endosomes does not occur *in vitro* in various systems (Gorvel *et al.*, 1991; Laurent *et al.*, 1998). Similarly, in *Leishmania* promastigotes, early endosomes prepared from fluid phase uptake of avidin and biotin-HRP do not fuse with late endosomes as observed in other systems. In contrast to this, early endosomes containing receptor mediated endocytic probe like BHb drive fusion both with early and late compartments in *Leishmania* (Figure 31) and this heterotypic fusion is regulated by Rab7 (Figure 32). Recently, a hemoglobin receptor from *Leishmania* has been reported (Sengupta *et al.*, 1999) which facilitates the uptake of hemoglobin by the promastigotes. Several studies have shown that the information for endocytosis and intracellular trafficking resides in the cytoplasmic domain of the receptor. For example, deletion of core kinase sequences from the distal region of the cytoplasmic domain of the EGF receptor impaired the proper trafficking of the receptor to the late/lysosomal compartment (Kornilova *et al.*, 1996). Similarly, it has been shown that a sequence distal to the endocytic motif of the cation-independent mannose 6-phosphate receptor (CI-MPR) in the cytoplasmic tail is required for efficient transport to the late endosomes (Juuti-Uusitalo *et al.*, 2000). In addition, DEC-205, an endocytic receptor like macrophage mannose receptor (MMR) expressed by dendritic cells, has a cytosolic domain containing coated pit localization sequences like MMR but targets to late endosomes (Jiang *et al.*, 1995). In contrast to MMR, DEC-205

receptor recycling occurs in the late compartment and the targeting signal for the late compartment is localized in the distal region of the cytoplasmic tail (Mahnke *et al.*, 2000). Thus, it is tempting to speculate that cytoplasmic tail of the hemoglobin receptor in *Leishmania* projecting from the early endosome transduces some signals to facilitate the fusion with late endosomes.

In order to unequivocally prove that cytoplasmic domain of the hemoglobin receptor regulates heterotypic fusion, we have expressed different deletion mutants of HbR (Figure 33), which is a transmembrane protein having kinase activity. Topology prediction (TMPred; Hofmann and Stoffel., 1993) of HbR sequence and the observed maximum binding of hemoglobin with HbR-ΔC suggest that possibly N-terminus is the extracellular domain of HbR (Figure 34). Our results show that BHb-loaded early endosomes pretreated with monoclonal antibody (1B6), specific to C-terminus of HbR, significantly inhibits heterotypic fusion with late endosomes containing AHRP (Figure 35). Similarly, addition of HbR-ΔN in the fusion assay inhibited about 80% of fusion whereas no significant inhibition was observed with HbR-ΔC or HbR-ΔNC. These results demonstrate that signals transduced from the HbR tail projecting from early endosomal compartment are blocked by C-terminus specific antibody or competed by HbR-ΔN, indicating that signals mediated through C-terminus cytoplasmic tail of HbR might promote the fusion with late endosomes.