Conclusion

6. Conclusion

Leishmania, like other trypanosomatid parasites, do not possess a complete heme biosynthetic pathway and must acquire heme from external sources. One of the potential sources of heme for the parasites could be hemoglobin. Previous studies from our laboratory have identified a receptor present on the surface of *Leishmania*, which mediates uptake of hemoglobin. Subsequently, internalized hemoglobin is targeted to the lysosomes where it is degraded probably to generate heme intracellularly. In the present thesis, we have designed experiments to understand the regulation of intracellular trafficking of hemoglobin in *Leishmania* promastigotes with special reference to their sorting mechanism in the early endosomal compartment.

To understand the intracellular trafficking of hemoglobin in *L. donovani* promastigotes and it's sorting in early endocytic compartment, we have used an *in vitro* reconstitution assay 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 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 mammalian cells. These results are in agreement with previous findings in higher eukaryotic cells that Rab5 positive early endosomes regulate early events of endocytosis, whereas Rab7, localized in late endosomes, serves as a targeting signal to the late compartments.

Our results also show that several features of homotypic early endosome fusion in *Leishmania* are similar to fusion events described previously in mammalian cells. Thus, early endosome fusion in *Leishmania* requires cytosol, ATP and its hydrolysis. Significantly higher level of fusion between endosomes from *Leishmania* is observed at 23°C than at 37°C. This may due the fact that this parasite optimally grows at 23°C and thus, all physiological processes like transport occur optimally at this temperature. This is not surprising since *Dictyostelium*, which grows optimally at 21-28°C exhibits significant fusion at similar temperature. It has been shown in several systems that Rab-GDI in presence of GDP specifically depletes Rab proteins from the membrane and exhibits broad substrate specificity across species. 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 is NSF, a homohexamer having both ATP binding and hydrolyzing activities. 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. NSF being an ATPase and 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. Accordingly, in our study, anti-mammalian NSF antibody specifically recognizes a ~70 kDa protein in *Leishmania* and fusion carried out in presence of cytosol immunodepleted using this antibody is significantly inhibited, demonstrating the role of NSF-like protein in endosome fusion in *Leishmania*. These results suggest that, as in higher eukaryotic cells, an NSF-mediated SNARE complex is likely to regulate endocytosis in *Leishmania*.

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 Rab5 homologue from *Leishmania*. BLAST search using LdRab5 sequence showed that cloned protein has about 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 demonstrated the presence of conserved Rab protein features including the GTP binding region, effector loop and C-terminal isoprenylation motif. These results suggest that cloned protein from *Leishmania* is a Rab5 homologue. The characteristic features of the Rab proteins are their GTP binding ability and intrinsic GTPase activity. Our results have shown that LdRab5 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 to its mammalian equivalent. However, LdRab5 contains RYKS and YYRGA, the signature motifs of Rab5, further confirming that the cloned protein from *Leishmania* is a Rab5 homologue. Immunolocalization studies show that Rab5 colocalizes with 5 min internalized hemoglobin-containing compartment indicating that Rab5 in *Leishmania* is localized in an early endocytic compartment. Subsequently, LdRab5, LdRab7 and specific antibodies were used to characterize endosome fusion in *Leishmania*. We observed that LdRab5 regulates fusion of biotinylated hemoglobin-loaded early endosomes with early endosomes containing avidin-HRP from *Leishmania*, resembling an earlier report in mammalian cells.

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*. Similarly, early and late endosomes, prepared from *Leishmania* promastigotes after fluid phase uptake of avidin and biotin-HRP, respectively, do not fuse as observed in other systems. In contrast, early endosomes containing receptor-mediated endocytic probe like biotinylated-hemoglobin 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 EGF receptor impaired proper trafficking of the receptor to the late/lysosomal compartment. Similarly, a sequence distal to the endocytic motif of CI-M6PR in the cytoplasmic tail is required for efficient transport to late endosomes. 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. Thus, it is tempting to speculate that the cytoplasmic tail of hemoglobin receptor projecting from the early endosome may transduce some signal(s) to mediate fusion with late endosomes in *Leishmania*.

In order to unequivocally prove that cytoplasmic domain of the hemoglobin receptor regulates heterotypic fusion, we have analyzed the role of different deletion mutants of hemoglobin receptor (HbR) like the N-terminus (HbR-ΔC), middle region

(HbR-ΔNC) and C-terminus (HbR-ΔN), recently cloned in our laboratory. Our results have shown that polyclonal antibody against hemoglobin receptor 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 with hemoglobin in comparison to other fragments. These results suggest that possibly N-terminus is the extracellular domain of hemoglobin receptor. Subsequent results have shown that biotinylated-hemoglobin loaded early endosomes pretreated with monoclonal antibody (1B6), specific to C-terminus of hemoglobin receptor, significantly inhibited heterotypic fusion with late endosomes containing AHRP. Similarly, addition of HbR-ΔN, a cytoplasmic tail specific fragment, 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 signal transduced from the hemoglobin receptor tail, projecting from early endosomal compartment is blocked by C-terminus specific antibody or competed by HbR-ΔN, indicating the signal mediated through C-terminus cytoplasmic tail of hemoglobin receptor may promote the fusion with late endosomes.

In conclusion, our results represent the first documentation that endocytosis in unicellular parasitic protozoa like *Leishmania* is regulated by small GTP binding proteins of 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 of membrane between the endosomes occurs. Subsequently, hemoglobin is targeted to the late/lysosomal compartment through signals mediated by the cytoplasmic tail of the receptor, which is Rab7 dependent.