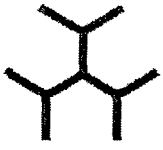
DETERMINATION OF THE FUNCTION OF A RAB PROTEIN IN INTRACELLULAR TRAFFICKING IN *Leishmania*



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NATIONAL INSTITUTE OF IMMUNOLOGY

CERTIFICATE

This is to certify that this thesis entitled " **Determination of the function of a Rab protein in intracellular trafficking in *Leishmania*** " by " **Sudha Bala Singh** " in partial fulfillment of the Ph.D. degree, of the **Jawaharlal Nehru University**, embodies the work done by the candidate under my guidance at the **National Institute of Immunology**. The work is original and has not been submitted in part or in full for any other degree or diploma of any university.

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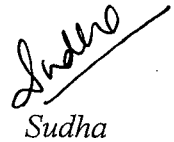


Sudha

CONTENTS

1. Introduction

2. Review of literature 4

2.1 Endocytosis 5

2.2 Clathrin mediated endocytosis 5

2.3 Intracellular trafficking 6

2.4 Rab proteins: regulators of intracellular transport 6

2.5 Characteristic structural features of Rabs 7

2.6 Localization of Rab proteins 9

2.7 Regulation of intracellular transport by Rab proteins 10

2.8 Regulation of intracellular trafficking in protozoa 16

2.9 Endocytosis and intracellular trafficking in trypanosomatid parasites 17

3. Chapter 1 20

3.1 Introduction 21

3.2 Materials and Methods 22

3.2.1 Materials 22

3.2.2 Antibodies and recombinant proteins 22

3.2.3 Cells 22

3.2.4 Cloning and expression of Rab5 from *Leishmania* 23

3.2.5 Expression and purification of LdRab5 protein 26

3.2.6 Protein estimation 26

3.2.7 SDS-PAGE and Western blot 26

3.2.8 Preparation of antibodies against LdRab5 27

3.2.9 GTP overlay assay 27

3.2.10 GTPase assay 28

3.2.11 Generation of LdRab5 mutants 28

3.3 Results 29

3.3.1 Cloning of Rab5 homologue from *Leishmania donovani* 29

3.3.2 Expression and purification of recombinant LdRab5 in *E.coli* 32
3.3.3 Specificity of anti-LdRab5 antibody 33
3.3.4 Characterization of recombinant LdRab5 34
3.3.5 Generation of LdRab5 mutants 35
3.3.6 Confirmation of the mutations by sequencing 36
3.3.7 Characterization of LdRab5 mutants 37
3.4 Discussion 39

4. Chapter 2 42
4.1 Introduction 43
4.2 Materials and Methods 43
4.2.1 Materials 44
4.2.2 Antibodies 44
4.2.3 Biotinylation of hemoglobin 45
4.2.4 Preparation of early endosomes from *Leishmania* 45
4.2.5 Subcellular fractionation of *Leishmania donovani* endocytic compartments 46
4.2.6 *In vitro* reconstitution of endosome fusion in *Leishmania* 46
4.2.7 Treatment of endosomes with GDI 47
4.2.8 Immunodepletion of Rab5 from *Leishmania* cytosol 47
4.2.9 *In vitro* prenylation recombinant Rab proteins 47
4.2.10 Immunolocalization of Rab5 in *Leishmania donovani* 48
4.3 Results 48
4.3.1 Subcellular fractionation of *Leishmania donovani* endocytic compartments 48
4.3.2 *In vitro* reconstitution of homotypic early endosome fusion 50
4.3.3 Role of NSF-like protein in endosome fusion in *Leishmania* 52
4.3.4 Role of endocytic Rabs in intracellular trafficking of Hb 53
4.3.5 Immunolocalization of Rab5 in *Leishmania donovani* 54
4.4 Discussion 55

5. Chapter 3 58

5.1 Introduction 59

5.2 Materials and Methods 60

5.2.1 Materials 60

5.2.2 Preparation of late endosomes from *Leishmania* 60

5.2.3 *In vitro* reconstitution of heterotypic endosome fusion in *Leishmania* 61

5.2.4 Immunodepletion of Rab5 and Rab7 from *Leishmania* cytosol 62

5.2.5 Treatment of hemoglobin containing early endosomes with anti-hemoglobin receptor antibody 62

5.2.6 Generation of hemoglobin receptor deletion mutants 62

5.2.7 Hemoglobin binding activity of the hemoglobin receptor and its deletion mutants 62

5.3 Results 63

5.3.1 Early and late endosomes in *Leishmania* differ in their biochemical composition 63

5.3.2 Comparison of heterotypic fusion between endososmes containing receptor-mediated or fluid phase probes 63

5.3.3 Role of Rab proteins in heterotypic fusion 64

5.3.4 Generation of different deletion mutants of hemoglobin receptor and their ability to bind hemoglobin 65

5.3.5 Role of hemoglobin receptor tail in heterotypic fusion 66

5.4 Discussion 67

6. Conclusions 69

7. Bibliography 74

ABBREVIATIONS

AHRP	Avidin- HorseRadish Peroxidase
ATP	Adenosine triphosphate
ATPγS	Adenosine 5'-0-thiotriphosphate.
ARF	ADP ribosylation factor
BCA	Bicinchoninic acid
BHb	Biotinylated hemoglobin
BSA	Bovine serum albumin
$CaCl_2$	Calcium Chloride
CD-M6PR	Cation dependent mannose 6 phosphate receptor
CI-M6PR	Cation independent mannose 6 phosphate receptor
cDNA	Complementary DNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNTPs	Deoxyribose nucleoside triphosphates
DTT	Dithiothreitol
Ci	Curie
°C	Degree Celsius
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediamine tetraacetic acid
EGTA	(Ethylene glyco-bis[β-aminoethyl ether]-N,N,N,'n'-tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ER	Endoplasmic Reticulum
FCS	Fetal calf serum
GAP	GTPases activating protein
GDF	Guanine nucleotide dissociation factor
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
GTPγS	Guanine 5'-3-0-(thio) triphosphate

HB	Homogenization buffer
Hb	Hemoglobin
HbR	Hemoglobin receptor
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
hr	Hour
HRP	Horse raddish peroxidase
IPTG	Isopropyl-beta-D-thiogalactopyranside
kDa	kilo Dalton
KCl	Potassium Chloride
LAMP	Lysosomal associated membrane protein
LB	Luria Bertani
µCi	micro Curie
M_r	Molecular weight
mg	milligram
min	minutes
µg	microgram
$MgCl_2$	Magnesium chloride
µl	microlitre
µM	micro Molar
ml	millilitre
mM	miliMolar
MEM	Minimum essential medium
NaCl	Sodium chloride
ng	nanogram
NHS	N-hydroxysuccinimide
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide sensitive fusion protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
PNS	Post nuclear supernatant .

RT-PCR	Reverse Transcription-PCR
SB	Solubilization buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGN	Trans Golgi network
t-SNARE	Target SNAP receptor
v-SNARE	Vesicle SNAP receptor

Introduction

1. Introduction

Leishmaniasis is caused by the protozoan parasite *Leishmania* and affects several million people worldwide (Herwaldt, 1999). The disease is manifested in pathologies that range from mild cutaneous lesions to fatal visceral Leishmaniasis, the outcome determined in part by the species of *Leishmania* and in part by host factors that are associated with the innate and acquired immune responses. Kala-azar, a chronic and often fatal form of human visceral Leishmaniasis is mainly found in Mediterranean Europe, South America particularly Brazil, North and east Africa, India, China and is predominantly caused by *Leishmania donovani.*

During its life cycle, *Leishmania* exist either as intracellular amastigotes within a specialized phagolysosomes of vertebrate macrophages or as extracellular promastigotes in the digestive tract of their vector, the sand fly. *Leishmania* and its close relative *Trypanosoma* represent a highly divergent eukaryotic lineage having many unique features at the molecular and cellular level. Compared to the mammalian cells, the organization and function of different intracellular organelles, especially the molecular machinery underlying secretory and endocytic processes of protozoa have not been studied in details.

A number of receptor systems like those for LDL, transferrin and fibronectin have been identified on the surface of trypanosomatids (Overath *et al.*, 1997) presumably, to endocytose essential nutrients. Recent studies from our laboratory have shown that hemoglobin endocytosis in *Leishmania donovani* is mediated through a 46 kDa protein located in the flagellar pocket (Sengupta *et al.*, 1999) probably to generate intracellular heme, as these parasites are unable to synthesize heme (Sah *et al.*, 2002). However, the intracellular route and mechanisms of transport in parasites remain to be explored.

Since endocytosis is the major route of entry of nutrient into cells, understanding the membrane trafficking events in *Leishmania* and their modulation by other signal transduction intermediates may provide new insights into the extent to which the basic machinery is conserved throughout evolution. However, endocytic mechanisms are largely inaccessible to direct biochemical manipulation because the component parts are located mostly in the cytoplasmic side of the membrane. The classical biochemical approach to unravel cellular mechanisms involved in protein trafficking is to develop a cell free assay and progress has been made in understanding the

regulation of intracellular trafficking of endocytosed molecules in higher eukaryotic cells using similar assays (Gorvel *et al.*, 1991). During the past few years, it has been shown that Rabs, which are GTP binding proteins of Ras superfamily, are versatile molecular switches that regulate intracellular transport through vesicle fusion.

Previous results have shown that GTP binding proteins of Ras superfamily are well conserved among different species and perform similar functions. It is reasonable to assume the existence of similar proteins in *Leishmania,* which may regulate intracellular trafficking of endocytosed molecules. Moreover, our previous results have shown that following initial binding of hemoglobin with its putative receptor localized on the cell surface of *Leishmania* promastigotes, hemoglobin is rapidly internalized into discrete intracellular vesicles, which possibly indicates that endocytosis and intracellular trafficking in *Leishmania* are regulated by vesicle fusion and fission.

In the present investigation, we have developed an *in vitro* reconstitution assay of vesicle fusion to understand the mechanism of intracellular trafficking of hemoglobin in *Leishmania.* We have shown that hemoglobin first enters into an early compartment of *Leishmania* promastigotes and is subsequently transported to the late compartment and this intracellular trafficking is regulated by specific Rab GTPases, cloned from *Leishmania,* through vesicle fusion. Moreover, our results represent the first documentation that the route of hemoglobin trafficking inside *Leishmania* is directed by the signals generated from the hemoglobin receptor tail.

Review of Literature

2.1 Endocytosis

Endocytosis has been studied extensively in the metazoans especially in mammalian cells, where it occurs by multiple mechanisms that fall into two broad categories, 'phagocytosis' or cell eating (the uptake of large particles) and 'pinocytosis' or cell drinking (the uptake of fluid and solutes). Phagocytosis is typically restricted to specialized mammalian cells such as macrophages for internalization of large particles (~500nm), bacteria, and other pathogens. In contrast to phagocytosis, pinocytosis usually internalizes macromolecules and small particles (<100nm).

Pinocytosis occurs in all cells by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Lamaze *et al.*, 2001). These diverse and highly regulated endocytic pathways control complex physiological processes such as hormone-mediated signal transduction, immune surveillance, antigen-presentation, and cellular and organismal homeostasis. Of the four known pathways of endocytosis, the clathrin-mediated pathway is by far the most studied one where mechanistic details of the pathway are much clearly known than the others.

2.2 Clathrin mediated endocytosis

Clathrin mediated endocytosis occurs constitutively in all mammalian cells, and regulates the uptake of essential nutrients, such as cholesterol-laden low-density lipoprotein (LDL) particles that bind to the LDL receptor and iron-loaded transferrin that binds to transferrin receptors (Schmid *et al.*, 1997; Brodsky *et al.*, 2001). These receptors are localized on specialized regions of the cell membrane, known as coated pits. Coated pits assemble at the plasma membrane following recruitment of coat proteins from the cytosol. Clathrin is the major component of the coat and is known to act as a scaffold (Brodsky, 1988). Clathrin is assmebled as a lattice of hexagons and pentagons (Pearse, 1987). The functional unit of these arrays is the triskelion, which consists of three 180 kDa clathrin heavy chains, each complexed with a 30-35 kDa light chain. A non-clathrin component required for the assembly of coats is the complex of adapter protein, which form an inner layer linking transmembrane receptors to the clathrin lattice (Vigers *et al.*, 1986). The adapter complex AP2 is found specifically at the plasma membrane, whereas AP1 is localized to the clathrin

coated vesicles that bud from the trans-Golgi network (Robinson and Pearse, 1986). With the aid of other accessory factors, the coat finally falls off the vesicle and allows the naked vesicle to fuse with the target membrane.

2.3 Intracellular trafficking

At the cell surface, coated pits containing clustered receptors along with their bound ligand bud from the plasma membrane and become coated vesicles. Following uncoating, these vesicles fuse with a peripheral population of endosomes, termed as early or sorting endosomes (early/sorting endosomes). In early/sorting endosomes, membrane receptors are sorted from their ligands depending on the particular receptor system. Some receptors are recycled to the cell surface directly (Yamashiro *et al.*, 1984; Ghosh *et al.*, 1994; Schmid *et al.*, 1988) or indirectly, through the perinuclear recycling endosomes (Hopkins, 1983; Mayor *et al.*, 1993). Alternatively, some receptors and ligands are transported to the late endosomes and lysosomes for degradation (Mellman *et al.*, 1986; Schmid *et al.*, 1988; Kornfeld and Mellman, 1989). Thus, the early endosome is the major sorting compartment from which subsequent transport takes place. Tubular regions of the endosomes are proposed to contain receptors destined to be recycled and vesicular regions contain material destined for degradation (Geuze *et al.*, 1983).

To ensure the proper progression of internalized cargo to the appropriate compartment and to maintain the functional identity of early/sorting endosomes, vesicle trafficking through this endocytic compartment must be tightly regulated. Evidences from a variety of sources, including temperature-sensitive mutants in yeast and *in vitro* reconstitution experiments have established that transport of cargo along the endocytic pathway requires a series of highly coordinated and specific vesicle fusion events regulated by small GTP binding proteins of the Rab family (Rothman and Sollner, 1997; Zerial and McBride, 2001; Pfeffer, 2001). Rab proteins are present on specific compartments and regulate transport of cargo molecules to the appropriate acceptor organelle(s) while maintaining the integrity of the membranes.

2.4 Rab proteins: regulators of intracellular transport

The Rab proteins belong to the superfamily of small (21-25 kDa) Ras related GTPases that consist of three major subfamilies, the Ras, Rho, and Rab proteins, as

well as the smaller ARF, Sar and Ran groups. The degree of amino acid identity between members of the different subfamilies is approximately 30% (Valencia *et al.*, 1991). All these proteins regulate membrane trafficking events by altering between two conformations in a nucleotide dependent fashion where, GTP bound form turns the protein "on" and hydrolysis of the GTP to GDP turns the protein "off".

The idea of Ras related GTPases being involved in membrane trafficking is based on the findings that conditional lethal mutations in *S.cerevisae* SEC4 and YPT1 genes led to defects in the secretory process. The Sec4 has shown to be localized to the cytoplasmic surface of secretory vesicles and plasma membrane and its mutants led to the accumulation of post-Golgi vesicles (Salminen and Novick, 1987, Goud *et al.*, 1988), whereas the related Ypt1p regulates transport step between ER and Golgi apparatus (Segev *et al.*, 1988). Tavitian *et al* have cloned the first homologues of SEC4/YPT in mammals, termed Rab (ras like in brain) genes. Today more then 60 Rabs are known in mammalian cells, which regulate different steps of membrane trafficking.

2.5 Characteristic structural features of Rabs

Structural information for the Rab proteins, obtained by X-ray crystallography is currently available for five members: mouse Rab3a (Dumas *et al.*, 1999; Ostermeier and Brunger, 1999), *Plasmodium falciparum* Rab6 (Chattopadhyay *et al.*, 2000), *S. cerevisiae* Ypt51p (Esters *et al.*, 2000) and Sec4p (Milburn *et al.*, 1990) and mouse Rab5C (Merithew *et al.*, 2001). Rab GTPases and Ras share a common fold, which consists of a six-stranded β sheet, comprising five parallel strands and one antiparallel, surrounded by five α helices. The amino acid residues responsible for guanine nucleotide and Mg^{2+} binding, as well as GTP hydrolysis are located in five loops that connect the α helices and β strands. The amino acid residues that come together in space to form this active site are closely associated with either the phosphate groups of the bound nucleotide and Mg^{2+} or the guanine base (Figure 1) and are highly conserved within the entire Ras superfamily. Crystallographic analysis of GDP and GTP bound states of the yeast Sec4p (Milburn *et al.*, 1990) show that major nucleotide-induced differences of the protein conformations occur in the switch I and switch II regions (Schlichting *et al.*, 1990).

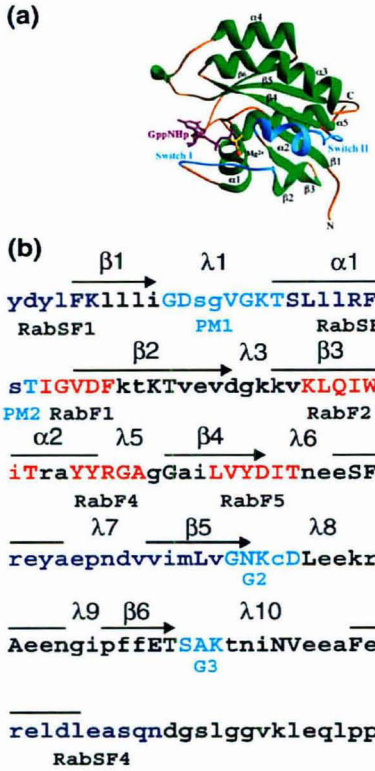


Figure 1. Structural features of Rab GTPases. **(a)** Ribbon drawing of Rab3A complexed with the GTP analog GppNHp. Purple, bound nucleotide; orange sphere, Mg^{2+} ion; blue, switch I and II regions; green, α helices and β sheets; yellow, loops. **(b)** A profile amino-acid sequence of the Rab GTPase subfamily generated using the hidden Markov model (HMM) method (Moore *et al.*, 1995). The uppercase/lowercase coding represents outcome of the profile HMM method. Upper case characters, residues found in the profile with a probability of $p > 0.5$; red, Rab-specific residues (RabF1-5); dark blue, subfamily-specific motifs (RabSF1-4); cyan, highly conserved nucleotide-binding motifs; G, guanine-base-binding motif; PM, phosphate/magnesium-binding motif. The secondary structure units (α helices, β strands, and loops, λ) are indicated above the sequence.

Adapted from Stenmark and Olkkonen., 2001.

These switch regions are located in the loop 2 region and the loop 4-α 2-loop 5 region, respectively, and in the three-dimensional structure, they are found on the surface of the molecule. Studies with appropriate mutations in these regions have shown that the putative switch regions are crucial for the interaction of Rab proteins with regulatory protein partners such as GDP/GTP exchange factors and GTPase-activating proteins. Similarly, structural studies have also indicated that these switch regions form an important part of the binding interface between Rab proteins and their effector molecules (Ostermeier and Brunger, 1999).

One of the key approaches to study Rab function has been the mutagenesis of specific amino acid residues essential for guanine nucleotide binding or GTP hydrolysis by the proteins. The effects of the mutant proteins have been determined morphologically and/or biochemically. The choice of amino acids to be mutagenized is based on well characterized mutations described in other GTPases, mainly the Ras oncoproteins. Earlier results have shown that substitution of leucine to glutamine in the WDTAGQE region (Q to L) in the PM3 conserved motif of Ras decreases the GTPase activity 10- to 100- fold and stabilizes the GTP bound, "active" conformation of the protein (Der *et al.*, 1986; Adari *et al.*, 1988; Stenmark *et al.*, 1994; Hoffenberg *et al.*, 1995). The steady state GTP hydrolysis rate by the mutant proteins is shown to

be less affected because the mutation also slows down the dissociation of GDP for the protein, which is the rate limiting step in the GTPase cycle (Hoffenberg *et al.*, 1995). In contrast, exchange of asparagine for serine (S to N) in the PM1 conserved motif of Ras displays lower affinity for GTP than GDP, locking the protein in inactive GDP bound form, leading to a dominant inhibitory effect (Feig and Cooper, 1988; Medema *et al.*, 1993). In addition to this, studies have also shown that deletion of the C-terminal motif of the Ras/Rab proteins results in failure of the mutant protein to attach to the target membrane, thereby inhibiting specific transport processes (Li and Stahl, 1993; Mukhopadhyay *et al.*, 1997).

2.6 Localization of Rab proteins

One of the hallmark properties of Rab GTPases is localization on distinct membrane compartments along the biosynthetic and endocytic pathways of eukaryotic cells. Some Rabs are expressed ubiquitously in human tissues, whereas others are tissue-specific. Within cells, they are localized to the cytosolic face of distinct intracellular membranes (Figure 2). Rab1 and Rab2 are found to be localized in ER and cis-Golgi, while, Rab6 and Rab9 are localized in medial Golgi and trans Golgi respectively (Chavrier *et al.*, 1990, Goud *et al.*, 1990). Though most of the Rabs have distinct membrane localizations, some like Rab5, localize to more than one compartment. Rab5 mainly localizes to early endosomes, but it is also present on the plasma membrane and on clathrin coated vesicles (Chavrier *et al.*, 1990), indicating the complex function of this protein. Moreover, Rab4, Rab5, Rab11, Rab18 and Rab24 have all been shown to be associated with early endocytic compartments, suggesting that the early endocytic compartment is highly complex with multiple functions (van der Slujis *et al.*, 1991; Bucci *et al.*, 1992; Li and Stahl, 1993). Among these, Rab4 appears to regulate the recycling from early endosomes to the plasma membrane, while Rab11 regulates recycling from perinuclear endosomes. In addition, late endosomes are positive for both Rab7 as well as Rab9 and the two proteins localize to different subdomains on the late endosomes (Barbero *et al.*, 2002) and perform different transport steps. Table-1 summarizes the localization of different Rabs proteins and their functions.

Name	Yeast homologue	Localization	Expression	Function
Rab1a	Ypt1p	ER/cis-Golgi	U	ER-Golgi transport
Rab2a		ER/cis-Golgi	U	Golgi-ER retrograde transport
Rab3a		SV	Neurons	Regulation of neuro-transmitter release
Rab4a		EE	U	Endocytic recycling
Rab5a	Ypt51p	EE, CCV, PM	U	Budding, motility and fusion in endocytosis
Rab6a	Ypt6p	Golgi	U	Retrograde Golgi traffic
Rab8a	Sec4p	TGN, PM	U	TGN-PM traffic
Rab9a		LE	U	LE-TGN traffic
Rab11a	Ypt31p	RE, TGN	U	Endocytic recycling via RE and TGN
Rab27a		Melanosomes Granules	Melanocytes Platelets Lymphocytes	Movement of lytic granules and melanosomes towards PM

Table-I: Localization and function of selected Rab GTPases. Abbreviations used for compartment: CCV, clathrin-coated vesicles; EE, early endosomes; ER, endoplasmic reticulum; LE, late endosome; PM, plasma membrane; RE, recycling endosome; SV, synaptic vesicle; TGN, *trans*-Golgi network k; U, ubiquitous.

2.7 Regulation of intracellular transport by Rab proteins

The membrane localization and subsequent biological functions of Rab proteins depends on the post-translational modification of a cysteine motif (CXXX, CC, CXC, CCXX or CCXXX where X is any amino acid) at the carboxyl terminus, by addition of one or two highly hydrophobic geranylgeranyl groups (Anant *et al.*, 1998). This post-translational modification requires the initial recognition of newly synthesized Rab protein by a Rab escort protein (REP), which presents the Rab

protein to the geranyl-geranyl transferase enzyme that prenylates the Rab protein through a cascade of molecular interactions (Seabra *et al.*, 1992; Andres *et al.*, 1993). REP then functions as a chaperone that keeps the hydrophobic, prenylated Rab soluble and delivers it to the appropriate membrane (Alexandrov *et al.*, 1994). A large number of studies have shown that deletion of C terminal cysteine residues of Rab proteins renders them inactive and the proteins fail to attach to the respective membrane.

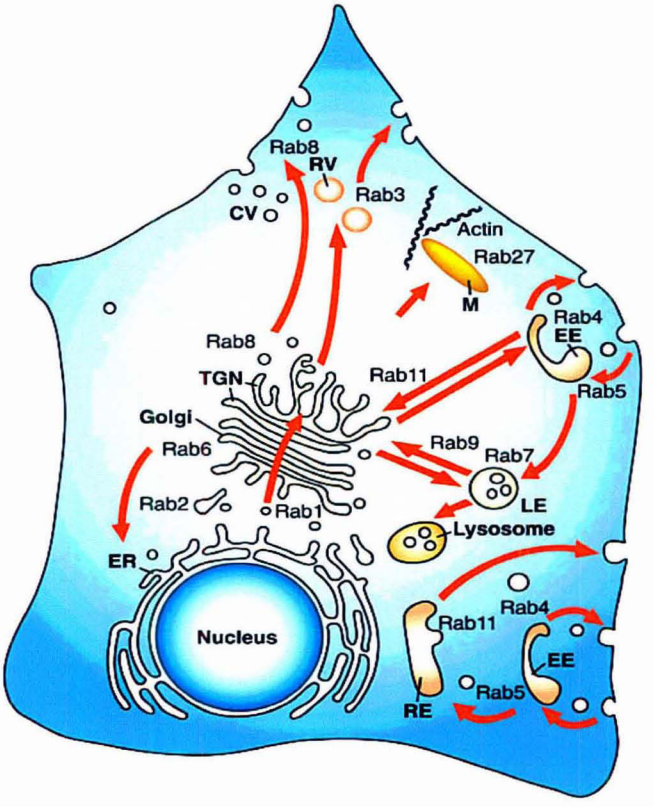


Figure 2. Intracellular vesicle transport pathways and localization of selected Rabs. The biosynthetic pathway, transport of proteins occurs from the endoplasmic reticulum (ER) through the Golgi complex to the cell surface. In endocytic pathway, material internalized from outside the cell, reaches the early endosomes (EE) first and can be recycled back to the surface, either directly or via a perinuclear recycling endosome (RE) compartment, or transported to late endosomes (LE) and lysosomes. The localization of selected mammalian Rab proteins in the membrane compartments participating in these transport processes is indicated.

Adapted from Stenmark and Olkkonen, 2001.

The REP-associated Rab GTPases are in the GDP-bound form. Subsequently, membrane delivery of these proteins is accompanied by the exchange of GDP with GTP, a reaction that is catalyzed by a GDP/GTP exchange factor (GEF). The GTP

form of Rab is unable to retain the REP (Alexandrov *et al.*, 1994). Following membrane fusion, GTP hydrolysis of Rab is catalyzed by a GTPase-activating protein (GAP), which converts it to GDP bound form. The GDP form of the Rab is retrieved by Rab GDP-dissociation inhibitor (GDI) from intracellular membranes (Ullrich *et al.*, 1993). GDI shares structural similarity with REP (Schalk *et al.*, 1996) and like REP, can present geranylgeranylated GDP-bound Rab proteins to specific membranes (Ullrich *et al.*, 1994). However, GDI is more abundant than REP and thus serves as a recycling factor that allows for several rounds of membrane association and retrieval of the Rab GTPases.

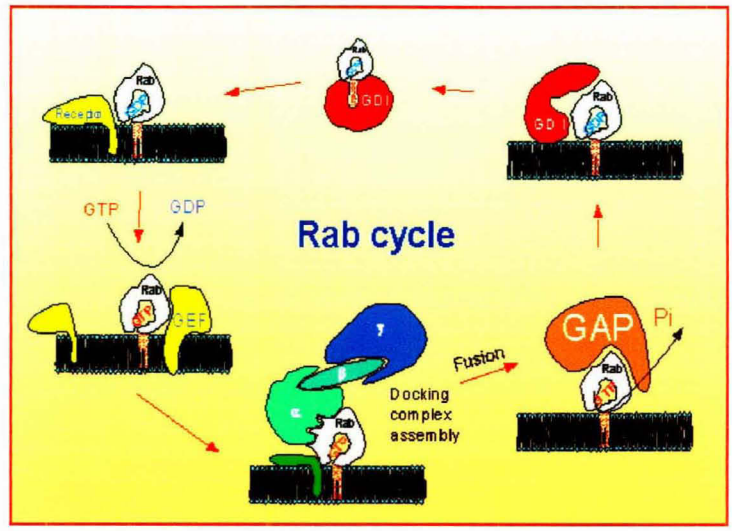


Figure 3. Rabs are molecular switches that oscillate between the GTP bound active conformation and GDP bound inactive state.

Evidences emerging from the most well characterized Rab protein, Rab5, show that the Rab GTPases are themselves tightly regulated by accessory proteins that modulate protein activity by controlling membrane association, nucleotide binding and hydrolysis (Mohrmann and van der Sluijs, 1999). The active form of Rab5 recruits various downstream effector molecules and tethering factors from the cytosol that bridge the specific vesicle with the target membranes. Rabaptin-5 was one of the first effectors to be identified for Rab5 (Stenmark *et al.*, 1995). The C terminus of Rabaptin-5 binds to Rab5 whereas the N terminus binds to Rab4, which has been implicated in the recycling of membrane from the sorting endosome to the cell surface. Hence, Rabaptin-5 may act in some way as a sensor to ensure that inward and

outward fluxes of membrane are balanced. Rabaptin-5 forms a complex with Rabex-5, the nucleotide exchange factor of Rab5 (Horiuchi *et al.*, 1997) and thereby induces recruitment of Rabex-5 on the membrane through Rabaptin-5 and catalyzes the conversion of Rab from GDP to GTP bound form. This GTP form of Rab5 on the membrane recruits other Rab5 effector molecules necessary for fusion.

One such molecule previously identified as early endosome autoantigen 1 (EEA1) (Simonsen *et al.*, 1998; Mills *et al.*, 1998), is essential for endosome fusion. EEA1 forms an extended homodimer (Callaghan *et al.*, 1999) and contains two Rab5 binding sites close to its N- and C-termini, both of which are flanked by zinc finger domains (Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999). Thus, EEA1 acts as a tethering molecule and helps in the docking of two endosomes during fusion. The C terminal zinc finger is closely related to other FYVE zinc finger motifs, found in other Rab5 associated proteins (Stenmark *et al.*, 1999) like Rabenosyn-5 (Nielsen *et al.*, 2000). FYVE fingers of Rabenosyn-5 bind to the lipid phosphatidylinositol-3-phosphate [PI(3)P] (Gaullier *et al.*, 1998; Patki *et al.*, 1998; Burd *et al.*, 1998) and provide membrane anchors for EEA1. While some of the effector molecules of Rab5 have been identified, however, recent studies indicate that there could be many more.

Recent studies have shown that similar effector molecules like tethering factors, exchange factors, GAP etc. of different Rabs are also present. These include Rab7, a late endocytic marker that is required for trafficking from early endosomes to late endosomes (Feng *et al.*, 1995; Meresse *et al.*, 1995). Recently, an effector of active Rab7 on late endosomes has been identified. This protein, termed RILP (for Rab7-interacting lysosomal protein), comprises of two coiled-coil regions typical of myosin-like proteins (Cantalupo *et al.*, 2001). RILP prevents the cycling of Rab7 and its expression induces the recruitment of functional dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes (Jordens *et al.*, 2001). Consequently, these compartments are transported by these motors toward the minus end of microtubules, effectively inhibiting their transport toward the cell periphery. Late endosomes are also positive for another Rab protein, Rab9 that regulates the trafficking of mannose-6-Phosphate (M6PR) receptors (Lombardi *et al.*, 1993). TIP 47 is a cytosolic protein that binds to M6PR cytoplasmic domains (Diaz and Pfeffer, 1998) as well as Rab9 (Carroll *et al.*, 2001). Binding of TIP47 to Rab9 triggers a conformational change of M6PR cytoplasmic domains, which regulates specific

transport steps (Carroll *et al.*, 2001). Table 2 represents a list of effector molecules known for different Rab proteins.

Rab	Rab function	Direct effector	Effector function	Rab specificity	Effector partners	Partner features
Rab1	• ER–Golgi transport	p115	• Tethering • Sequestering SNAREs into budded vesicles	Rab1–GTP	Giantin GM130	• Tethering of COPI-coated vesicles to Golgi
		PRA1	• Rab receptor (proposed)	Rab1 Rab3 Rab4b Rab5a Rab5c	VAMP2	• v-SNARE involved in bilayer fusion
Rab3	• Rab3a: synaptic vesicle and chromaffin granule secretion • Rab3b, c, d: regulated secretion	Rabphilin-3	• Potentiates fusion	Rab3–GTP	α-actinin Rabaptin-5	• Crosslinks actin filaments into bundles • Stimulated by Rabphilin-3 interactions • Also binds Rabaptin-5, an effector of Rab5 and Rab4
		RIM1 RIM2	• Membrane fusion	Rab3–GTP	RIM–BP1	• Contains fibronectin type III repeats and SH3 domains
		Calmodulin	• Confers calcium sensitivity to protein interactions	Rab3	Many	• Multiple functions
Rab4	• Localized to early/recycling endosomes • Role in sorting/recycling in early endosomes	Rabaptin-5, Rabaptin-5β Rabaptin-4	• Activates Rab5 through complex with Rabex-5 • Implicated in protein sorting and recycling	Rab4–GTP Rab5–GTP	Rabex-5	• Nucleotide exchange factor
Rab5	• Ligand sequestration at plasma membrane • CCV–EE and EE–EE fusion • Endosome motility	Rabaptin-5 Rabaptin-5β	• Stabilizes Rabex-5 recruitment	Rab5–GTP Rab4–GTP	Rabex-5 Rabaphilin-3	• Nucleotide exchange factor
		EEA1	• Tethering, core fusion component	Rab5–GTP	Syntaxin13 Syntaxin6	• t-SNAREs essential for bilayer fusion
		p150	• Class III PI(3)K regulatory subunit	Rab5–GTP	hVps34	• Class III PI(3)K catalytic subunit
		p110β	• Class I PI(3)K catalytic subunit	Rab5–GTP	p85-α	• Class I PI(3)K regulatory subunit
		Rabenosyn-5	• Required for CCV–EE and EE–EE fusion	Rab5–GTP Rab4–GTP	hVps45	• Regulates SNARE complex formation or disassembly
Rab6	• Retrograde Golgi–ER and intra-Golgi transport	Rabkinesin-6	• Vesicle motility • Cytokinesis	Rab6–GTP	Microtubules	
Rab8	• TGN–plasma membrane traffic (basolateral in epithelial cells)	Rab8IP	• Stress-activated protein kinase	Rab8–GTP		
Rab9	• Late endosome to Golgi	p40	• Stimulates fusion	Rab9–GTP		
Rab11	• Recycling through perinuclear recycling endosomes • Plasma membrane–Golgi traffic	Rab11BP	• Unclear	Rab11–GTP	mSec13	• Coat component of COPII vesicles
Rab13	• Involved in the formation of the tight junction	δ-PDE	• Extracts Rab13 from membrane	Rab13		
Rab33b	• Intra-Golgi transport	Rab33b-BP	• Probably regulates motility of Rab33 vesicles	Rab33b–GTP		

Table 2 Rab protein and their effector molecules. Abbreviations used for compartment: CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; PDE, phosphodiesterase; PI(3)K, phosphoinositol-3-OH kinase; SH3, Src homology region 3 domain;TGN, *trans*-Golgi network; Vamp, vesicle-associated membrane protein.
Adapted from Zerial and McBride, 2001.

A second level of specificity in membrane trafficking events is regulated by soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNARES). SNAREs comprise a large family of proteins (Jahn and Sudhof, 1999) whose members are found predominantly on either target membranes (t-SNARES), or on donor vesicles (v-SNAREs) (Sollner *et al.*, 1993). Members of the v- and t-SNARE families are localized to distinct sub-cellular compartments (Advani *et al.*, 1998). SNAREs function in virtually all membrane trafficking events and are conserved across broad evolutionary distances (Ferro-Novick and Jahn, 1994). A cascade of protein–protein interactions is required to ensure that SNARE partners are

made available to each other appropriately, in order to maximize the fidelity and efficiency of transport reactions. Firstly, 'cis'-SNARE complexes, the products of previous fusion reactions that consequently contain both v- and t-SNAREs within the same membrane, must be disassembled. This task is performed by the soluble co-factor SNAP (soluble NSF attachment protein), which specifically binds NSF (Nichols and Pelham, 1998; Brennwald, 2000). Binding of these proteins to cis-SNARE complexes (Weber *et al.*, 1998; McNew *et al.*, 1999) is followed by NSF-dependent ATP hydrolysis that uncoils the core complex so that SNAREs are released from each other (Brennwald, 2000; Weber *et al.*, 1998). This is accompanied by a conformational change in the t-SNARE that prevents it from rebinding to its partner v-SNARE (Parlati *et al.*, 1999). The unpaired t-SNARE is further stabilized by a member of the Sec1p family of proteins (Poirier *et al.*, 1998; Chen *et al.*, 1999). Incoming vesicles initially interact with the target membrane via a specific tether molecule, which is unique to each transport event, in conjunction with Rab proteins. Subsequently Sec1 is released by activated Rab, driving the t-SNARE into an open conformation, which eventually interacts with a specific cognate v-SNARE, in order to promote membrane fusion as illustrated in Figure 4.

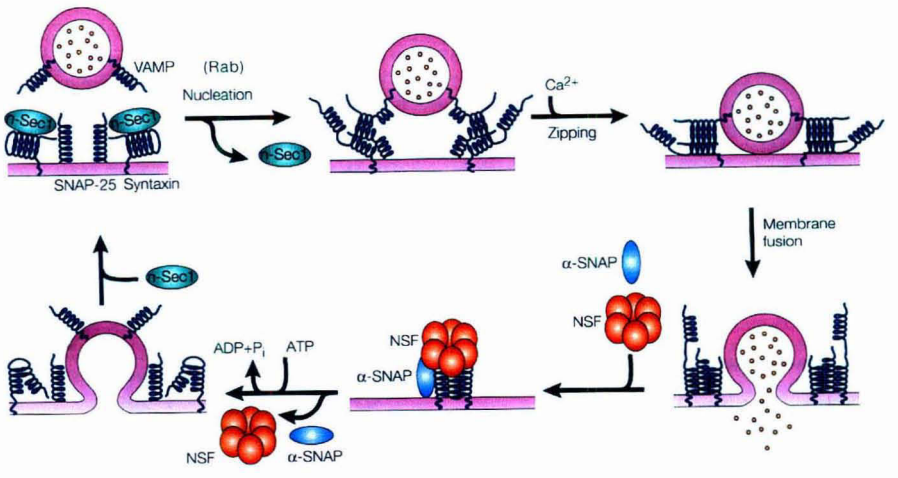


Figure 4. Molecular model of vesicle fusion. VAMP denotes vesicle associated v-SNARE; SNAP-25, 25-kDa synaptosome associated protein; Syntaxin, a t-SNARE in exocytosis; SNAP, soluble NSF attachment protein. Adapted from Chen and Scheller, 2001.

2.8 Regulation of intracellular trafficking in protozoa

Given the important role Rab proteins play in controlling intracellular trafficking in cells, various groups have started exploring the presence of these proteins in protozoan parasites. Recent studies indicate that different Rab homologues are also present in a wide variety of protozoan parasites. These studies are mainly based on their identification and localization rather than elucidating their role in vesicular transport. A Rab6 homologue has been identified in *Toxoplasma gondii* (Stedman *et al.*, 2003) where it regulates retrograde transport from the post-Golgi secretory granules. Similarly, Rab6 homologue present in *Plasmodium falciparum* is also associated with the Golgi network (de Castro *et al.*, 1996). In the endocytic pathway of *Toxoplasma gondii*, Rab5 homologue is shown to localize in tubulovesicular structures anterior to the parasite nucleus and facilitates the uptake of cholesterol possibly through a Golgi dependent pathway (Robibaro *et al.*, 2002). As seen in mammalian cells, late endosomal Rabs like Rab7 regulate phagosome maturation in *Dictyostelium discoidium*, (Rupper *et al.*, 2001). However, in contrast to previous studies, Rab7 regulates homotypic fusion of early endosomes in this organism. In addition, Rab4 and Rab11, which are involved in recycling in mammalian cells, have also been identified in *Dictyostelium* (Temesvari *et al.*, 1994; Adesi *et al.*, 1995). Rab11, which is associated with the perinuclear recycling endosomes in mammalian cells, has been observed to be associated with the contractile vacuole in *Dictyostelium* (Harris *et al.*, 2001).) In *Entamoeba histolytica*, another protozoan parasite, it is demonstrated that Rab11 and Rab7 are localized to the endosomal compartments (Temesvari *et al.*, 1999; Welter *et al.*, 2002). Moreover, *P. falciparum* homologue of Rab GDI has also been identified (Attal and Langsley, 1996) indicating that cycling of Rab from cytosolic to membrane bound form also occurs in this parasite. These observations suggest that endocytic and biosynthetic pathways in some unicellular protozoa is regulated by Rab GTPases as observed in higher eukaryotic cells.

Other ubiquitous molecules required for fusion machinery have also been identified in protozoan parasites, such as N-ethylmaleimide sensitive factor from *Toxoplasma* (Liendo *et al.*, 2001), *Dictyostelium* (Weidenhaupt *et al.*, 1998) and *Paramecium* (Kissmehl *et al.*, 2002). Membrane fusion specificity is further regulated by appropriate SNARE molecules. Recent studies have shown that early endosome

fusion in *Dictyostelium* involves a Syntaxin 7 homologue, an early endosoma SNARE (Bogdanovic *et al.*, 2000). These results indicate that NSF-mediated SNARE complexes are likely to regulate the transport in unicellular protozoa, as in higher eukaryotic cells.

2.9 Endocytosis and intracellular trafficking in trypanosomatid parasites

The family Trypanosomatidae (order Kinetoplastida) comprises of numerous protozoan genera, none of which are free living. Some of these cause important disease in humans (Herwaldt, 1999). These include *Trypanosoma brucei,* the causative agent of African sleeping sickness and nagana in cattle; *T. cruzi,* the causative agent of chagas disease and *Leishmania* species, the causative agent of visceral and muco-cutaneous Leishmaniasis throughout the tropics and subtropics. These protozoan parasites have digenetic life cycle that exist in two distinct morphological forms, intracellular amastigotes in the vertebrate host and extracellular promastigotes/epimastigotes in the insect vector (Killick, 1979; Chang and Dwyer, 1976). The promastigote form can be easily grown under appropriate culture conditions and thus, this form of the parasites is widely used to study biochemical and metabolic pathways in the protozoan parasites.

In both eukaryotic and prokaryotic cells, 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 like *Trypanosoma* and *Leishmania* (Clayton *et al.*, 1995; Liu *et al.*, 2000). One distinctive feature of this group of parasites in contrast to mammalian cells is that endocytosis occurs exclusively through a specialized region of the cells, the flagellar pocket, located at the base of flagella in the anterior end (Langreth and Balber, 1975; Webster and Russell, 1989; Weise *et al.*, 2000). Few receptor systems have been identified in trypanosomatid parasites, which mediate uptake of low-density lipoprotein and transferrin presumably for efficient supply of nutrients such as cholesterol and metal ions required for rapid growth (Voyiatzaki and Soteriadou, 1992; Bastin *et al.*, 1996). Recent studies from our laboratory have demonstrated that endocytosis of hemoglobin in *Leishmania* is mediated through receptors located in the flagellar pocket (Sengupta *et al.*, 1999), possibly to generate intracellular heme after

degradation of internalized hemoglobin, as *Leishmania* lack a complete heme biosynthetic pathway (Sah *et al.*, 2002). However, the intracellular route and mechanism of transport of internalized materials in protozoan parasites remains largely unknown.

Endocytosis in trypansomatids is known to be restricted to the flagellar pocket. The early endosomes commonly consist of a system of pleiomorphic tubules and cisternal structures that are invariably localized around the flagellar pocket and in close proximity to the Golgi apparatus (Langreth and Balber, 1975; Webster and Russell, 1989). However the rate of endocytosis varies enormously in different trypanosomatids. Endocytosis is mediated primarily by a population of clathrin coated vesicles that bud from the flagellar pocket and deliver a range of fluid phase markers and membrane markers to a system of cisternal elements and collecting tubules (Langreth and Balber, 1975; Morgan *et al.*, 2001). This tubulovesicular complex contains distinct populations of endosomes, as defined by the distribution of several Rab protein homologues. Plasma membrane proteins and flagellar pocket receptors that are internalized into the tubulovesicular endosomes may be recycled back to the flagellar pocket or transported to the late endosome/lysosome compartments (Brickman *et al.*, 1985; Duszenko *et al.*, 1988; Hager *et al.*, 1994; Webster, 1989).

Recently, homologues of Rab4, Rab5, Rab7 and Rab11 have been identified in *Trypanosoma brucei.* Two Rab5 isoforms have been detected in *Trypanosoma brucei*, TbRab5A and TbRab5B (Field *et al.*, 1998). TbRab5B is involved in the trafficking of transmembrane receptors, whereas TbRab5A regulates trafficking of GPI anchored receptors. TbRab4 and TbRab11 are localized on the structures in contact with the TbRab5A compartment and are potentially involved in the recycling pathways (Field *et al.*, 1998). In *Leishmania*, two Rab homologues have been identified. LmYPT is a mammalian Rab1 homlogue and is localized on the Golgi apparatus (Cappai *et al.*, 1993), as in mammalian cells. Concurrent with our studies, a Rab7 homologue in *L. major* has been recently identified and shown to be associated with late endocytic/lysosomal compartment (Denny *et al.*, 2002).

Considerable progress has been made in the last few years in defining the ultrastructure of the secretory and endocytic pathways of trypanosomatids. These studies have been greatly facilitated by the identification of organelle-specific markers and more recently, the identification of genes (via genome searches) that regulate intra- and inter-organelle transport. Collectively, these studies demonstrate that the

basic features of the trypanosomatid secretory and endocytic pathways are very similar to those found in other eukaryotes, despite the fact that these organisms represent one of the most divergent eukaryotic lineages. In this respect, the trypanosomatids may prove to be interesting experimental systems for investigating different aspects of protein and lipid trafficking. On the other hand, some aspects of the trypanosomatid secretory and endocytic pathways are clearly unusual. Interestingly, transport in the secretory pathway of *T. brucei* and *Leishmania* is not inhibited by Brefeldin A, a potent inhibitor of COPI coat formation in many animal cells (Engel *et al.*, 2000; Figueiredo and Soares, 1995), suggesting that one or more proteins involved in ER-Golgi transport in trypanosomatids differ from their mammalian counterpart. Thus, any major differences observed in the endocytosis and intracellular trafficking mechanisms in these pathogenic protozoa as compared to the mammalian host cells can be exploited for therapeutic intervention.

Chapter 1

Cloning and expression of Rab5 from Leishmania donovani

3.1 Introduction

Rab GTPases represent a broad family of Ras-related proteins, which localize to a particular set of intracellular compartments. These proteins specifically regulate docking and fusion between two distinct vesicles (McLauchlan *et al.*, 1998; Christoforidis *et al.*, 1999) during intracellular transport in order to deliver the cargo to the right destination. Among 63 different Rab proteins identified so far, Rab5 is the best-characterized endosomal Rab that is shown to regulate membrane traffic into and between early endosomes (Gorvel *et al.*, 1991; Mukhopadhyay *et al.*, 1997). Like any other Rab GTPases, Rab5 regulates fusion between early endosomes in a nucleotide dependent manner. Strong evidence for the role of Rab5 in endosome fusion comes from the observation that activated, GTP-locked mutant of Rab5 stimulates docking and fusion whereas GDP-locked mutant is inactive (Gorvel *et al.*, 1991; Li *et al.*, 1994). Recent studies using the activated mutant show that at least 20 different cytosolic proteins specifically interact with Rab5, highlighting the complexity of the downstream regulation of this GTPase (Zerial and McBride, 2001).

The first such effector identified for Rab5 is Rabaptin-5 (Stenmark *et al.*, 1995) that forms a complex with Rabex-5, which catalyses nucleotide exchange on Rab5 (Horiuchi *et al.*, 1997). Activated Rab5 along with the Rabaptin-5-Rabex-5 complex induces its own membrane recruitment through Rabpatin-5. This creates a microenvironment on membrane that is enriched in activated Rab5 where other Rab5 effectors are recruited. Another effector, early endosome autoantigen1 (EEA1), essential for endosome fusion was previously identified (Simonsen *et al.*, 1998; Mills *et al.*, 1998). EEA1 contains two Rab5 binding sites, close to its amino and carboxyl termini, both of which are flanked by zinc finger domains (Simonsen *et al.*, 1998). Thus, EEA1 bridges two early endosomal compartments containing Rab5. The C-terminal zinc finger of EEA1 containing FYVE motif binds to phosphatidylinositol-3-phosphate [PI(3)P] (Gaullier *et al.*, 1998; Patki *et al.*, 1998; Burd *et al.*, 1998) and phosphatidylinositol kinases preferentially phosphorylate [PI(3)P], which helps EEA1 to anchor to the membrane (Toker and Cantley, 1997). Additionally, Rabenosyn-5, another FYVE finger Rab5 effector also stabilizes the early endosome docking complex in a similar way (Nielsen *et al.*, 2000; Patki *et al.*, 1998). Recent studies have shown that Rab5 also stimulates both the association of early endosomes with microtubules and the movement of early endosomes (Nielsen *et al.*, 1999).

Given the important role Rab5 has in controlling early endosome dynamics in mammalian cells, it would be imperative to identify a Rab5 homologue in *Leishmania*, which would facilitate an understanding of the nature and regulation of the endocytic pathway in *Leishmania*. Recently, two Rab5 isoforms have been identified in *T. brucei*, TbRab5A and TbRab5B (Field *et al.*, 1998). TbRab5B is involved in the trafficking of transmembrane receptors, whereas TbRab5A regulates trafficking of GPI anchored receptors. Nonetheless how these Rab proteins regulate endocytosis and what are the major players involved in this process, are not known. In order to understand the mechanism of hemoglobin trafficking and sorting in early endosomal compartment in *Leishmania*, we have cloned, expressed and characterized a Rab5 homologue from this organism in the present investigation.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co (St. Louis, MO). RT-PCR kit was procured from GIBCO BRL, (Gaithersburg, MD). pGEM-T-easy cloning vector was purchased from Promega Life Science (Madison WI). Expression vector pGEX 4T-2 and ECL reagents were procured from Amersham Bioscience (Amersham UK). All the secondary antibodies labeled with HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents used were of analytical grade. Molecular biology reagents were procured from Promega Life Science and Amersham Bioscience.

3.2.2 Antibodies and Recombinant Proteins

Recombinant mammalian Rab 5 fusion protein was kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St Louis, MO). A mouse monoclonal anti-Rab5 antibody recognizing mammalian Rab5 was received as a gift from Dr. A. Wandinger-Ness (Northwestern University, Evanston, IL).

3.2.3 Cells

Promastigotes of *Leishmania donovani* (UR 6) were obtained from Indian Institute of Chemical Biology, Kolkata, India. Cells were routinely maintained on solid blood agar slants containing glucose, peptone, sodium chloride, beef heart

extract, and rabbit blood with gentamicin (Roy, 1932). For experimental purposes, cells were harvested from 3 day old blood agar slants by scraping into phosphate buffered (10mM, pH 7.2) saline (0.15M).

3.2.4 Cloning and expression of Rab5 from *Leishmania*

In order to clone Rab5 from *Leishmania*, BLAST search was carried out using *Trypanosoma brucei* Rab5B sequence as a query. One putative Rab5-like sequence was identified from *Leishmania major* genome having substantial homology with TbRab5B and Rab5 from other sources. Accordingly, primers were designed against start and stop codons of the putative Rab5 sequence of *L. major*. The forward and reverse primers were synthesized containing *Bam*HI and *Eco*RI restriction sites, respectively. The sequence of forward primer was 5′-GGATCCATGTCATCCATCAGTCGC-3′ and reverse primer was 5′-GAATTCCTAGCAGCATCCGTTCTCT-3′. RT- PCR was performed using these primers to amplify the ORF of the putative Rab5 sequence using *L. donovani* cDNA. First, mRNA was isolated from *Leishmania* promastigotes using Oligotex mRNA kit (Qiagen, Germany). Subsequently, cDNA was synthesized using Thermo Script RT-PCR kit (GibcoBRL). Briefly, 1.5 μg of mRNA in nuclease-free water was mixed with 2 μl of Rab5 reverse primer, 10 μl 5X RT buffer, 1 μl RNase inhibitor (40 units/ μl), 2.5 μl 0.1 M DTT, 2 μl of dNTPs (10 mM each) and 0.5 μl of reverse transcriptase enzyme (15 units/ μl). The mixture was incubated at 37°C for 1 hr and then heat inactivated for 5 min at 90 °C. Subsequently, PCR was performed using Rab5 forward and reverse primers in a Perkin-Elmer thermocycler for 30 cycles: denaturation for 1 min at 94 °C; annealing at 55°C for 30 sec and extension was carried out for 1 min at 72 °C. A ~650 bp fragment was amplified using Rab5 specific primers. *Taq* polymerase used for this PCR is known to incorporate untemplated 3' A overhangs, which facilitated cloning of the PCR product into pGEM-T-easy vector, which has T-overhangs. Subsequently, positive clones were sequenced using m13 universal primers in an automated sequencer. After confirming the sequence, the PCR products were further sub-cloned into *Bam*HI/*Eco*RI sites of pGEX-4T-2 expression vector and transformed into XL-1Blue *E.coli* (Stratagene).

Figure 5a. Schematic representation of the cloning of *Leishmania* Rab5 into pGEM-T-easy vector

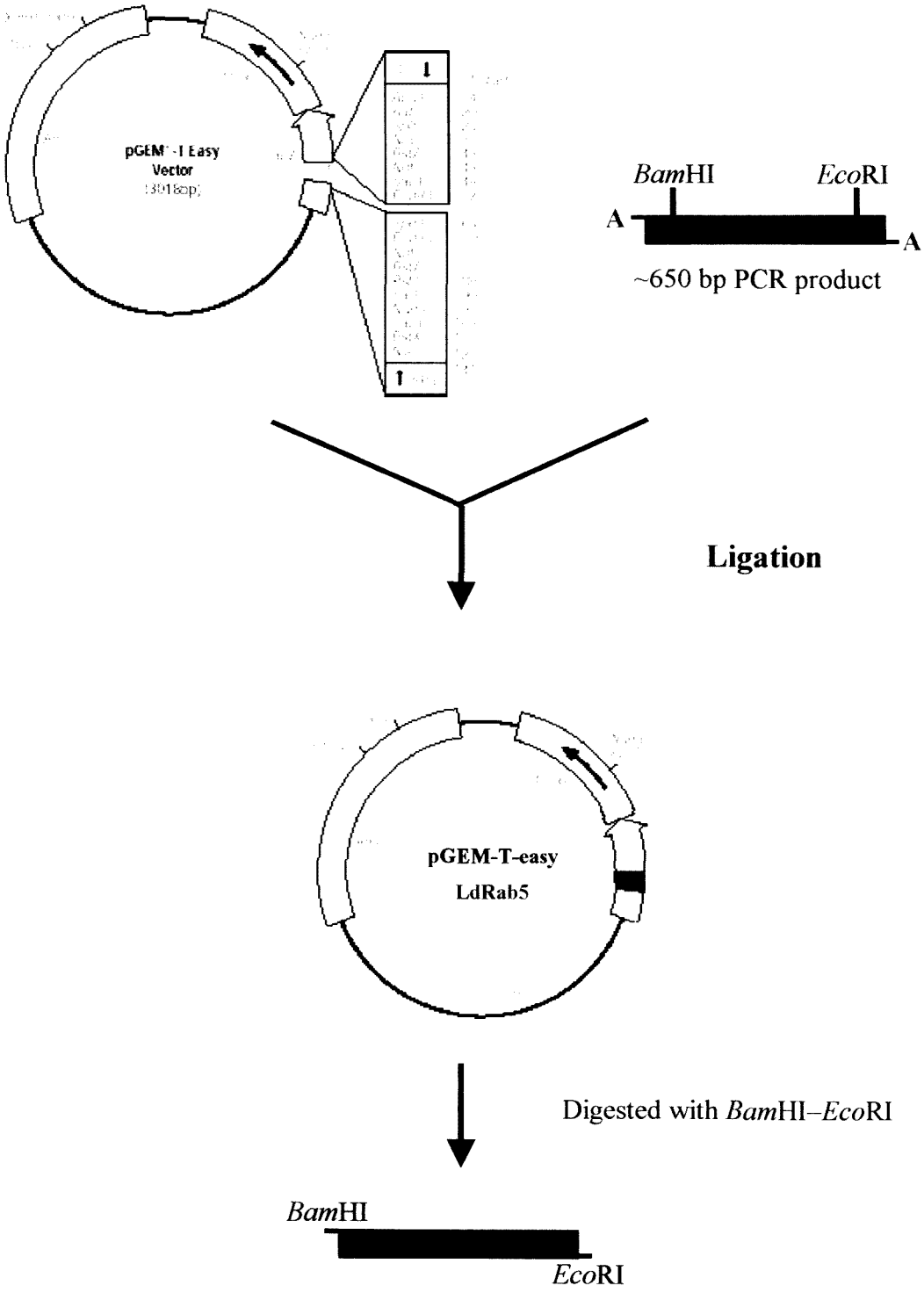
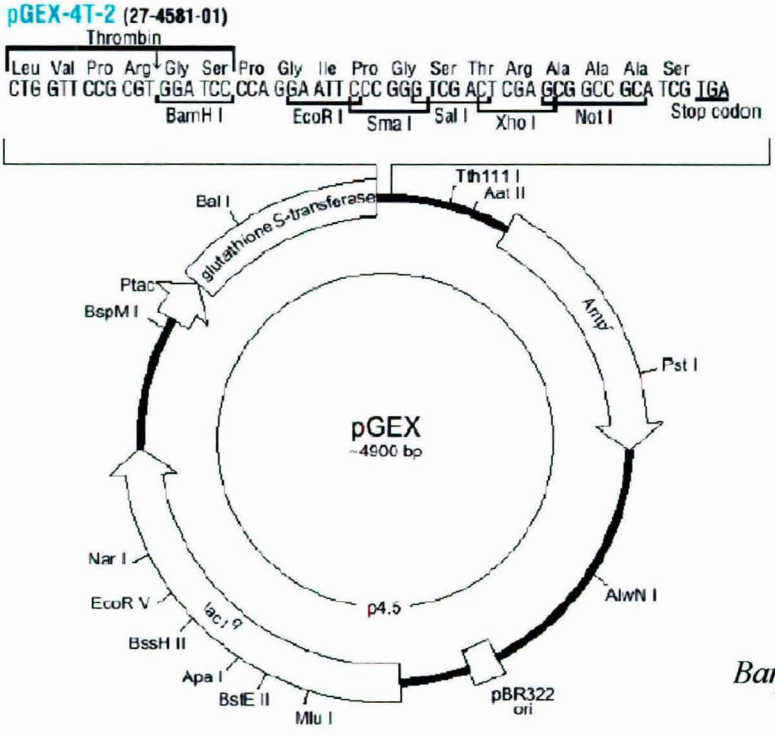
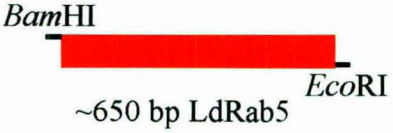


Figure 5b. Schematic representation of the sub-cloning of *Leishmania* Rab5 into pGEX-4T-2 vector

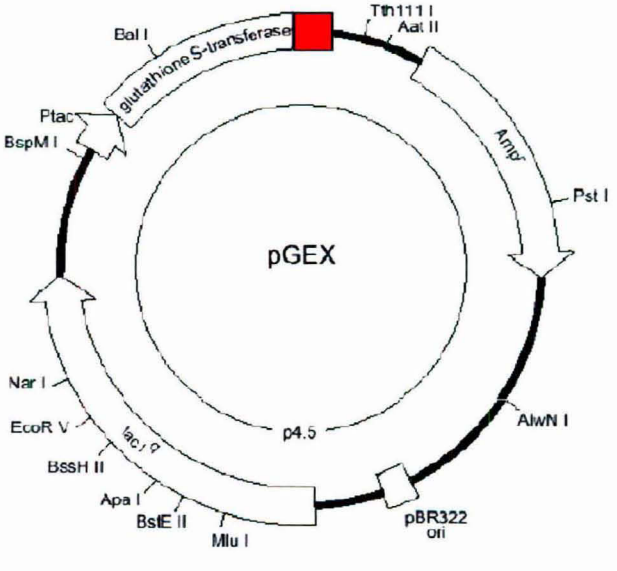


pGEX-4T-2 vector digested with *Bam*HI-*Eco*RI



~650 bp LdRab5

Ligation



3.2.5 Expression and purification of LdRab5 protein

E.coli transformed with pGEX-4T-2:LdRab5 plasmid was grown to O.D. $_{600}$ of 0.5 in LB and subsequently induced with 0.2 mM IPTG for 3 hr at 37°C in order to express recombinant *L. donovani* Rab5 (LdRab5). Cells were harvested and treated with lysozyme (1mg/ml) for 30 mins at room temperature for lysing the cells. Following treatment, DTT was added to the lysate to a final concentration of 1mg/ml. Subsequently, cells were subjected to sonication and the lysate was treated with Triton X-100 (1%). Unbroken cells and other debris were separated by centrifugation at 18000 g for 10 min at 4°C. The GST fusion protein (GST-LdRab5) was purified from the supernatant using glutathione beads by standard procedure.

3.2.6 Protein estimation

Protein estimations were carried out as per the manufacturer's instructions using the Bradford protein detection assay and bicinchoninic acid (BCA) protein assay kit (Pierce, USA).

3.2.7 SDS-PAGE and Western Blot

The protein profiles were analyzed by SDS-PAGE. The samples were resuspended in SDS sample buffer (0.0625 M Tris, pH-6.8, 2% SDS, 10% glycerol, 5% β mercaptoethanol, and 0.001% bromophenol blue), boiled for 5 min, resolved on a 12% acrylamide gel and visualized by coomassie staining. For Western blotting, the gel was soaked in Tris-Glycine buffer (25 mM Tris, 200 mM glycine, containing 20% methanol) for 15 min. Nitrocellulose membrane (0.45 μm; BioRad, Hercules, CA, USA) was also presoaked in the same buffer before transfer. The proteins were then electrophoretically transferred to the nitrocellulose membrane at a constant voltage of 10V for 30mins, using a Trans-Blot SD semi-dry transfer cell (BioRad). Subsequently, the membrane was stained with Ponceau to check for the efficiency of protein transfer. The membrane was washed once for 5 min with phosphate-buffered saline (PBS; 50 mM Phosphate and 150 mM NaCl, pH 7.4) and blocked in 5% BSA-PBST (PBS containing 0.1% Tween-20) at 4°C overnight. Thereafter, the membrane was washed thrice with PBST and incubated with the respective primary antibody for 1 hour at room temperature. The membrane was washed thrice with PBST and incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 hr

at room temperature. The blot was developed with ECL reagent (Pharmacia) according to manufacturer's protocol and the signals obtained were captured on photographic film (Hyperfilm ^{TM} MP, Amersham Pharmacia Biotech, UK).

3.2.8 Preparation of antibody against LdRab5

GST-LdRab5 immobilized on glutathione beads was treated with 10 units of thrombin (Pharmacia) in cleavage buffer (50mM Tris-HCl, pH 7.5 containing 150 mM NaCl and 2.5mM $CaCl_2$) for 3 hr at room temperature to cleave the LdRab5 from GST tag (Smith and Corcoran, 1993). LdRab5 released after cleavage was separated from the immobilized GST by centrifugation. The purity of the released LdRab5 was checked by SDS-PAGE. Subsequently, mice were immunized with the cleaved LdRab5 to raise antibody by standard techniques (Overkamp *et al.*, 1988). Specificity of the antibody was determined by ELISA and Western blot analysis using LdRab5 recombinant protein and *Leishmania* cytosol (40ug/lane). Antibody against LdRab7 was also generated following a similar approach, using LdRab7 recently cloned in our laboratory.

3.2.9 GTP overlay assay

In order to determine the ability of purified LdRab5 to bind GTP, different concentrations of GST-LdRab5 and its respective mutants, along with mammalian GST-Rab5 proteins were blotted onto nitrocellulose membranes. The membranes were incubated with PBST for 2 hrs at room temperature followed by four washes with 50 mM phosphate buffer, pH 7.5 containing 5 mM $MgCl_2$. Subsequently, GTP-binding state of the Rab5 was detected by incubating with 1μCi/ml of [α-^{32}P] GTP (3000 Ci/mM, NEN) in GTP binding buffer (50 mM phosphate buffer, pH 7.5 containing 5 mM $MgCl_2$, 1 mM EGTA and 0.3% Tween-20) for 3 hrs at room temperature as described previously (Via *et al.*, 1997). Finally, the membranes were washed five times with 50 mM phosphate buffer, pH 7.5 containing 5 mM $MgCl_2$ to remove unbound [α-^{32}P] GTP and visualized by autoradiography. To determine the specificity of GTP binding, similar experiments were carried out in the presence of 1mM unlabeled GTP, GDP and ATP.

3.2.10 GTPase assay

The GTPase activity of LdRab5 and its mutants along with mammalian GST-Rab5 proteins were determined using the procedure described previously (Stenmark *et al.*, 1994). Briefly, 2 pmol of immobilized indicated Rab5 was incubated with buffer A (20mM Tris-HCl, pH7.8, 100mM NaCl, 5mM $MgCl_2$, 1mM Na-phosphate and 10mM 2-mercaptoethanol) for 20 min at 25°C and bound nucleotide was eluted with 1M guanidine–HCl. Immobilized proteins were then incubated with 2 pmol α- ^{32}P GTP (800 Ci/mmol, Perkin Elmer Life Sciences, MA) in 20µl buffer A for 10 min at 0° C followed by 1hr at 23°C. Samples were washed and incubated in 8µl of buffer B (0.2%SDS, 2mM EDTA, 10mM GDP, 10mM GTP, pH7.5) and heated for 2 min at 70 °C. An aliquot was analyzed using thin layer chromatography and visualized by autoradiography.

3.2.11 Generation of LdRab5 mutants

To determine the role of *Leishmania* Rab5 more precisely, three mutants *viz*, GTP-locked (Q80L), GDP-locked (S34N) and isoprenylation deficient (ΔC), were generated by PCR using appropriate primers. In order to generate the ΔC mutant, PCR was carried out using wild type forward primer (5'-GGATCCATGTCATCCATCAGTCGC-3') and mutant reverse primer (5'-CCG GAATTCCTATCCGTTCTCTTGGCG-3') in which nucleotides corresponding to the two C-terminal cysteine residues were deleted. The PCR was carried out using 50 ng of LdRab5 (WT) as template under conditions identical to those described previously.

In order to generate the Q80L mutant of LdRab5, a primer was designed such that the Q residue at position 80 was changed to L residue {i.e. CAG codon was changed to CTN (A/C/G/T)}. The mutation was introduced by making use of PCR-based mutagenesis. In the first round of PCR, a megaprimer was amplified using the forward mutant primer (5'-ACGGCAGGGCTNGAGCGCTTT-3') and the reverse wild type primer (5'-GAATTCCTAGCAGCATCCGTTCTCT-3') from 30 ng of LdRab5 (WT) as template. PCR was carried out for 30 cycles of denaturation at 94 °C for 20 sec, annealing at 56°C for 20 sec and extension at 72°C for 1 min. The amplified megaprimer (~400bp) was gel purified using QiaGen Gel purification kit. Subsequently, a second PCR was carried out in order to amplify the full-length mutant product (~ 650 bp). LdRab5 (WT) was used as template with the forward wild type

primer (5'-GGATCCATGTCATCCATCAGTCGC-3') and the megaprimer as reverse primer. The PCR was set up as follows: 5 cycles of denaturation at 94°C for 1 min and extension at 72 °C for 1 min to allow synthesis of the megaprimer strand. While at 72°C, 25 pmoles/μl of the Rab5 forward primer was added and PCR was performed over 25 cycles of denaturation at 94 °C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. Likewise, the S34N mutant of LdRab5 was generated using a similar procedure as that described for Q80L. The mutant primer was designed such that the TCG codon of serine at position 34 was changed to AAY (G/C) of asparagine. The megaprimer was amplified using the forward mutant primer (5'-GTCGGCAAG AAYTCTGTCGTG-3') and the reverse wild type primer as discussed above. Subsequently, the full-length S34N mutant was generated using this megaprimer by the same procedure as described for the generation of Q80L mutant of LdRab5. The full-length PCR products (~650bp) were subsequently cloned into pGEM-T-easy vector and sequenced to confirm the respective mutations. The products were further sub-cloned into the *Bam*HI/*Eco*RI sites of pGEX4T-2 and expressed as GST fusion proteins.

3.3 Results

3.3.1 Cloning of Rab5 homologue from *Leishmania donovani*

In order to clone the Rab5 homologue from *L. donovani*, BLAST search was carried out using *Trypanosoma* Rab5B sequence as a query, which revealed a putative

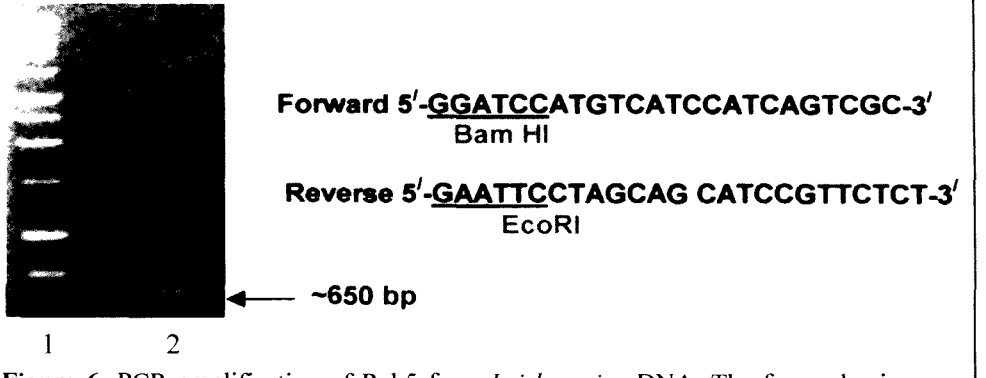


Figure 6. PCR amplification of Rab5 from *Leishmania* cDNA. The forward primer including *Bam*HI and reverse primer including *Eco*RI were designed against the putative Rab5 sequence that is available in *Leishmania major* genome database. A band of ~650 bp was amplified (Lane2). Lane 1: 1 kb DNA ladder (Promega).

Rab5-like sequence from *L. major* genome with 72% homology. Appropriate forward and reverse primers were used to amplify a fragment (~650bp) from *L. donovani* cDNA using PCR (Figure 6). The PCR product was cloned into pGEM-T-easy vector and sequenced by automated sequencing using m13 universal forward and reverse primers (Figure 7a).

a. Putative LdRab5 nucleotide sequence (636 bp)

atgtcatcca tcagtcgcgc ctcgacgacg acggcggggg gttccgcttc
gacacggaag ttcaagctag tcctgctcgg ggagagtggc gtcggcaagt
cgtctgtcgt gcagcgtctc atgaagaacg cctttagcga gaagctgaac
agcaccgtcg gcgcctcctt cttccggtac acgtgcaacg tggacgacga
caccgccgtc cacttcgata tatgggacac ggcagggcag gagcgcttta
agagtttagc ctctatgtac taccgcggag ctgctgcggc gctcgtcgtg
ttcgacattg tctccgccga tacgttcgaa aaggcaaggt actggattcg
agagctgcaa gccaactcgc cagagacgat tgtgatgctg gtaggcaaca
agaaggactt ggagagcgag cggcaggtgt ctgtggcaga tgcgcagcag
tgcgcggtgg agatgggtgc cgtgtaccac gagactagcg cgcgcagcgg
cgacggggtt cgggatgcat tccacgccgt cgctgcaaaa ctgatcgaga
caaacagtgc cttcagcgtg cgcgagggcg gtgtcatgtg ccacacagag
aacgcggcac cgcgccaaga gaacggatgc tgctag

b. Putative LdRab5 amino acid sequence (211 aa)

MSSISRASTT TAGGSASTRK FKLVLLGESG VGKSSVVQRL MKNAFSEKLN
STVGASFFRY TCNVDDDTAV HFDIWDTAGQ ERFKSLASMY YRGAAAALVV
FDIVSADTFE KARYWIRELQ ANSPETIVML VGNKKDLESE RQVSVADAQQ
CAVEMGAVYH ETSARSGDGV RDAFHAVAAK LIETNSAFSV REGGVMCHTE
NAAPRQENG C C*

(conserved domains of Rab proteins)

Figure 7. The PCR product was cloned into pGEM-T-easy and sequenced. The nucleotide sequence (a) was hypothetically translated into amino acid sequence (b). The amino acid sequence reveals the presence of conserved GTP binding domains (highlighted in pink).

The nucleotide sequence was hypothetically translated (211 amino acids) (Figure 7b) and BLAST search of non-redundant databases identified the cloned protein as Rab5 homologue from *L. donovani,* which was designated as LdRab5. Analysis of LdRab5 protein sequence demonstrates the presence of conserved canonical Rab protein features (Olkkonen and Stenmark, 1997) like GTP-binding region, effector loop and C-terminal isoprenylation motif etc.

Multiple sequence alignment of Rab5

```
LdRab5          MSSISRAS----------TTTAGGSASTRKFKLVLLGESGVGKSSVVQRLMKDAFSEKLN 50
TbRab5          MSVKTVA-----------------APTKKYKIVLLGDSGVGKSSLVQRLAKNEWCDNQN 42
MusRab5         --MTSRS--------TARPNGQPQASKICQFKLVLLGESAVGKSSLVLRFVKGQFHEYQE 50
DmRab5          MATTPRSGGASGTGTAQRPNGTSQN-KSCQFKLVLLGESAVGKSSLVLRFVKGQFHEYQE 59
Ypt51           ----------------------MNTSVTSIKLVLLGEAAVGKSSIVLRFVSNDFAENKE 37

LdRab5          STVGASFFRYTCNVDDDTAVHFDIWDTAGQERFKSLASMYYRGAAAALVVFDIVSADTFE 110
TbRab5          STGRRVFLRYVCTVG-DTAVNFDIWDTAGQERYKSLASMYYRGAAAALVVYEIPSWETFE 101
MusRab5         STIGAAFLTQSVCLD-DTTVKFEIWDTAGQERYHSLAPMYYRGAQAAIVVYDITNQETFA 109
DmRab5          STIGAAFLTQTICIE-DTVVKFEIWDTAGQERYHSLAPMYYRGAQAAIVVYDIQNQDSFQ 118
Ypt51           PTIGAAFLTQRVTIN-EHTVKFEIWDTAGQERFASLAPMYYRNAQAALVVYDVTKPQSFI 96

LdRab5          KARYWIRELQANSS-ETVVMLVGNKKDLE---SERQVSLADAQQCAGEMGAMYHETSARS 166
TbRab5          RAKHWVRELATNSP-ETIVILVGNKSDLRG--TSGCVSSEEAATYARELNLLFSEASAKD 158
MusRab5         RAKTWVKELQRQASPSIVIALAGNKADLAN---KRMVEYEEAQAYADDNSLLFMETSAKT 166
DmRab5          RAKTWVKELHKQASPNIVIALAGNKADLSN---IRVVEFDEAKQYAEENGLLFMETSAKT 175
Ypt51           KARHWVKELHEQASKDIIIALVGNKIDMLQEGGERKVAREEGEKLAEEKGLLFFETSAKT 156

LdRab5          GDGVRDAFHAVAAKLIEANSTFNVREGG-----VMCHTENAAPRAEN--GCC-- 211
TbRab5          GSGVSEVFMQIAQRLVASNNNNTVHSGG-----VLGGQPNSTRRSS---GCCG- 203
MusRab5         AMNVNDLFLAIAKKLPKSEPQNLGGAAG----RSRGVDLHEQSQQNKS-QCCSN 215
DmRab5          GMNVNDIFLAIAKKLPKNDGANNQGTSI----RPTGTETNRPTNN-----CCK- 219
Ypt51           GENVNDVFLGIGEKIPLKTAEEQNSASNERESNNQRVDLNAANDGTSANSACSC 210
```

Figure. 8. Multiple alignment of amino acid sequence of LdRab5 with Rab5 sequences from different organisms. Residues implicated in guanine phosphate binding (PM1-3), GTP/GDP binding (G1-3) and isoprenylation motif (I) are marked in bold face. 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*.

Alignment and comparison of LdRab5 sequence with other Rab5 sequences using CLUSTAL W software (Thompson *et al.*, 1994) demonstrated that LdRab5 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.

A putative structure was predicted for LdRab5 (Figure 9), by homology-based modeling (Swiss-model software) using mouse Rab5c as a template (Merithew *et al.*, 2001). The predicted structure indicates that LdRab5 shares motifs characteristic of Rabs, such as six β-sheets, comprising five parallel strands and one antiparallel strand, surrounded by five α-helices. Subsequently, a phylogeny of the Rab5 lineage was constructed using GCG package, on various sequences of Rab5 identified from different organisms (Figure 10). Irrespective of mammalian or protozoan origin both Rab5 and Rab7 originate from a common ancestor, Ras, but subsequently diverge.

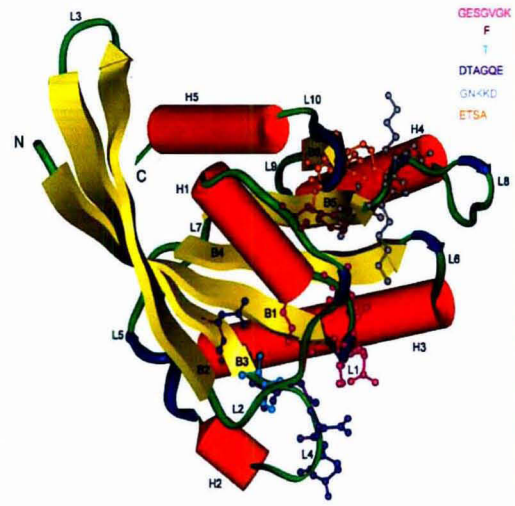


Figure 9. Knowledge based modeling (Swiss-model software) of LdRab5 showing characteristic nucleotide binding motifs consisting of six-stranded β sheet (yellow) surrounded by five α helices (red).

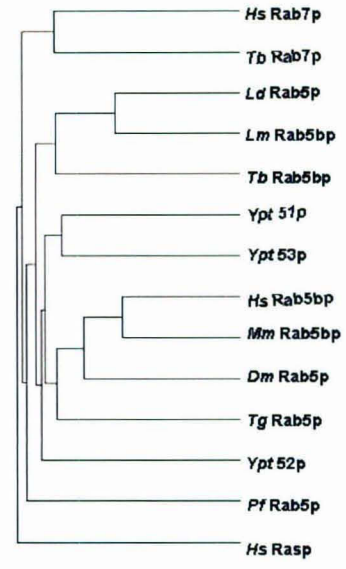


Figure 10. Phylogenetic relationship between Rab5 of different organisms. Tree was generated using GCG package on various sequences of Rab5 identified from different organisms. Tb, *T. brucei*; Mm, *M. musculus*; Dm, *D. melanogaster*; Ypt, *S. cerevisiae*; Hs, *H. sapiens*; Ld, *L. donovani*; Lm, *L. major*; Tg, *T. gondii*; Pf, *P. falciparum*.

L. donovani and *L. major* Rab5 co-segregate as a monophyletic group and their close homologue is Rab5 from *T. brucei*.

3.3.2 Expression and purification of recombinant LdRab5 in *E.coli*

In order to express recombinant LdRab5, *E.coli* transformed with pGEX-4T-2:LdRab5 plasmid were grown and induced with IPTG. Cells were lysed and proteins were extracted with Triton X-100. Finally, unbroken cells and other debris were separated and the GST-Ldrab5 fusion protein from the supernatant was purified using glutathione beads. Yield of the LdRab5 in each purification step was monitored by SDS-PAGE analysis. Results presented in Figure 11 show that LdRab5 is expressed

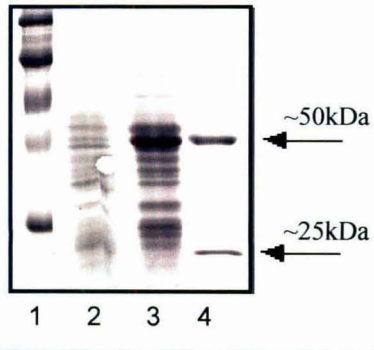


Figure 11. SDS-PAGE profile shows LdRab5 at different stages of purification. Lane1, molecular weight maker; Lane2, uninduced cells; Lane3, induced sample; Lane 4, purified fusion protein.

by IPTG induction and the fusion protein of expected size (~50-kDa) can be purified to homogeneity using glutathione-agarose beads. The lower band corresponding to ~25 kDa obtained along with LdRab5 fusion protein (~50 kDa), is probably free GST.

3.3.3 Specificity of anti-LdRab5 antibody

To examine the intracellular localization of Rab5 in *Leishmania*, specific antibody was raised against recombinant LdRab5. The anti-LdRab5 is specific and does not cross react with GST. Western blot analyses presented in Figure 12a show that antibody raised against LdRab5 specifically recognized *Leishmania* protein but not mammalian Rab5.

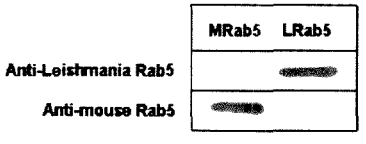


Figure 12a. Specificity of *Leishmania* Rab5. To determine specificity of LdRab5, mammalian Rab5 (MRab5) or LdRab5 (1 μg protein each per lane) were electrophoresed and transferred to nitrocellulose membranes. Subsequently, membranes were incubated with appropriate dilution (1:2000) of specific antibodies against mammalian Rab5 or *Leishmania* Rab5 as indicated. The proteins were visualized using appropriate HRP-conjugated second antibodies and ECL. Results from Western blots are representative of three independent preparations.

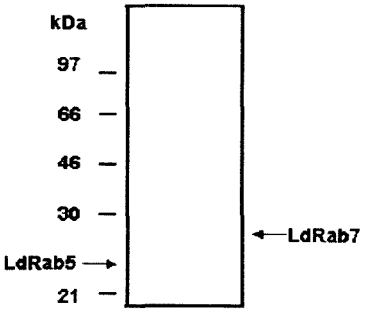


Figure 12b. Specificity of *Leishmania* Rab5 and Rab7 antibodies. *Leishmania* cell lysate (40 μg/ lane) was analyzed by Western blot using specific antibodies against LdRab5 and LdRab7. Results of Western blots are representative of three independent preparations.

Similarly, a monoclonal antibody against the mouse Rab5 does not cross react with *Leishmania* Rab5. Recently, we have cloned and expressed Rab7 from *L. donovani* and raised antibody against *Leishmania* Rab7 which specifically recognized a ~26-kDa protein from *Leishmania* lysate. However, the antibody raised against LdRab5 specifically recognized ~23 kDa protein in *Leishmania* cytosol (Figure 12b). These results indicate that the antibodies against the respective proteins are very specific and do not cross react with either protein.

3.3.4 Characterization of recombinant LdRab5

To determine GTP binding ability as well as GTPase activity of LdRab5, GTP blot analysis and GTPase activity were carried out with the GST-LdRab5 fusion protein using free GST and mammalian GST-Rab5 as controls. The results presented in Figure 13a show that LdRab5 fusion protein could bind significant amount of [α^{32}P]-GTP. No GTP binding was observed with free GST indicating the specificity of GTP binding to LdRab5. In order to determine the specificity of guanine nucleotide binding ability of LdRab5, protein was incubated with the labeled GTP, in the presence of unlabeled GTP, GDP or ATP. The binding of labeled GTP was competed out by addition of either 1mM GTP or 1mM GDP but not by 1mM ATP, indicating that LdRab5 specifically binds to guanine nucleotides (Figure 13b).

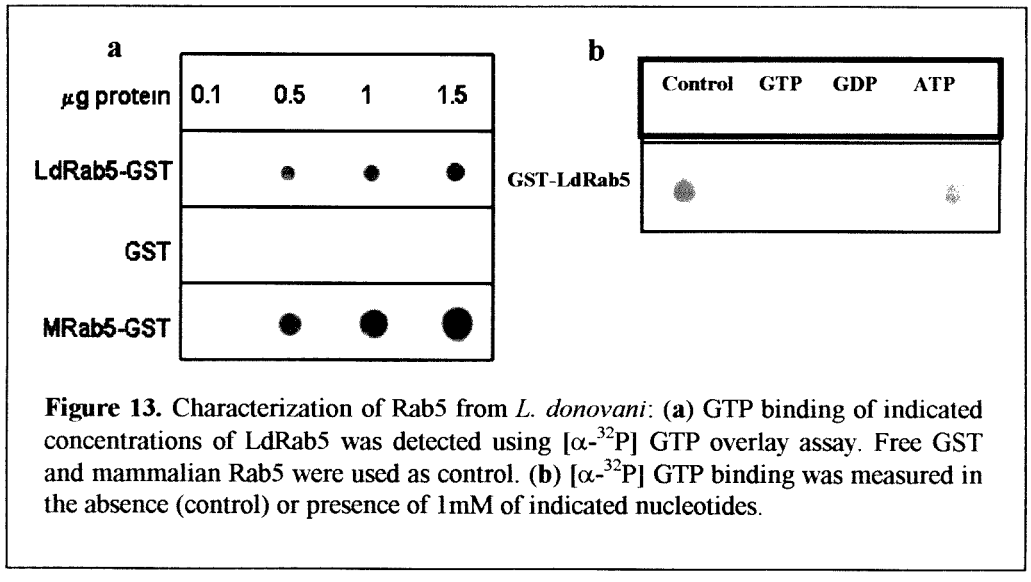


Figure 13. Characterization of Rab5 from *L. donovani*: (**a**) GTP binding of indicated concentrations of LdRab5 was detected using [α-^{32}P] GTP overlay assay. Free GST and mammalian Rab5 were used as control. (**b**) [α-^{32}P] GTP binding was measured in the absence (control) or presence of 1mM of indicated nucleotides.

GTP hydrolysis or intrinsic GTPase activity is known to be another major property of this group of proteins. Subsequently, GTP hydrolysis of LdRab5 was measured and compared with the that of the well-characterized mammalian Rab5:WT protein. Rab5:Q79L, a GTP-locked mutant of Rab5 with reduced GTPase activity, was used as control. The kinetics of GTP hydrolysis profile in Figure 14 showed that the mammalian Rab5:WT hydrolyzed most of the GTP by 1hr, whereas GTP hydrolysis of LdRab5 was much slower and only 50% GTP hydrolysis was observed even after 90 min. No GTP hydrolysis was detected with Rab5:Q79L mutant mammalian Rab5 protein, further confirming the reduced GTPase activity of this mutant protein.

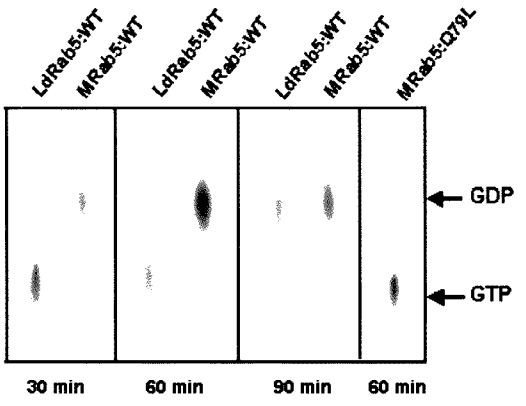


Figure 14. Kinetics of GTPase activity of LdRab5 was determined as stated in Materials and Methods. Mammalian Rab5 wild type (WT) protein and Rab5:Q79L, a GTP locked mutant, were used as control. Results are representative of three independent preparations.

3.3.5 Generation of LdRab5 mutants

The LdRab5 mutants were generated using PCR based mutagenesis. Accordingly, the ΔC mutant was amplified using a reverse mutant primer, which would introduce a C-terminal deletion of two cysteine residues. These residues are required for prenylation of the protein and their subsequent association with membrane. Our results showed that a ~650 bp fragment was amplified using these primers and wild type LdRab5 as template (Figure 15, lane2). The GDP and GTP-locked mutants of LdRab5 were generated using megaprimer based mutagenesis. Earlier evidences showed that conversion of asparagine to serine in GKT/S region of Rab proteins render them constitutively locked in the GDP form. Accordingly, a mutant forward primer was designed to replace serine at position 34 by asparagine. Subsequently, PCR was performed on wild type LdRab5 template, using this forward primer along with wild type reverse primer, which amplified a ~530bp fragment containing the desired mutation (Figure 15, lane 3). The obtained fragment was gel purified and this megaprimer was used as reverse

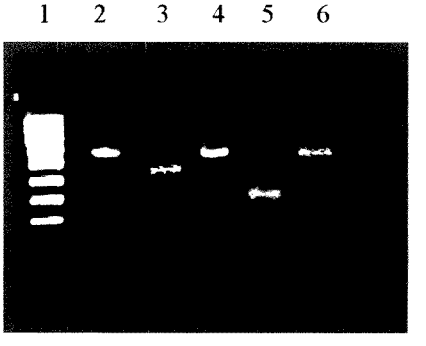


Figure 15. The LdRab5 mutants were generated using mutant primers as described in materials and methods. Lane1 represents 1 kb DNA ladder (Promega). Lane2 shows the PCR amplification for ΔC mutant. Lane3: represents PCR amplification of S34N megaprimer (~530bp). Lane 4: S34N full-length product. Lane 5 shows the megaprimer amplified for the Q80L mutant. Lane 6:Full-length PCR product of Q80L mutant. The samples were run on 1.2% agarose gel.

primer for the second PCR along with wild type forward primer to generate the full-length product (Figure11, lane 4). Using a similar approach, the GTP-locked LdRab5 was generated where the first primer was designed in such a way that glutamine at position 80 is replaced by Leucine. In the first round of PCR, megaprimer of ~400bp was amplified with the mutant forward primer and reverse wild type primer (Figure 15, lane 5). Subsequently, the full-length gene containing Q80L mutation was amplified with the megaprimer and the original forward primer (Figure 15, lane 6).

3.3.6 Confirmation of the mutations by sequencing

The PCR products for the mutants were cloned into pGEM-T-easy vector and sequenced. The sequencing data confirmed introduction of appropriate changes in the respective mutants (Figure 16). Sequence of LdRab5:ΔC showed that the codons, tgc

atgtcatcca tcagtcgcgc ctcgacgacg acggcggggg gttccgcttc
gacacggaag ttcaagctag tcctgctcgg ggagagtggc gtcggcaaga
actctgtcgt gcagcgtctc atgaagaacg cctttagcga gaagctgaac
agcaccgtcg gcgcctcctt cttccggtac acgtgcaacg tggacgacga
caccgccgtc cacttcgata tatgggacac ggcaggg[illegible] gagcgcttta
agagtttagc ctctatgtac taccgcggag ctgctgcggc gctcgtcgtg
ttcgacattg tctccgccga tacgttcgaa aaggcaaggt actggattcg
agagctgcaa gccaactcgc cagagacgat tgtgatgctg gtaggcaaca
agaaggactt ggagagcgag cggcaggtgt ctgtggcaga tgcgcagcag
tgcgcggtgg agatgggtgc cgtgtaccac gagactagcg cgcgcagcgg
cgacggggtt cgggatgcat tccacgccgt cgctgcaaaa ctgatcgaga
caaacagtgc cttcagcgtg cgcgagggcg gtgtcatgtg ccacacagag
aacgcggcac cgcgccaaga gaacggatgc tgctag

Figure 16. The color-coded LdRab5 nucleotide sequence showing the changes of nucleotide sequence in respective mutant. Serine (tcg) at position 34 of the wt protein has been changed to asparagine (aac) in the S34N mutant, glutamine (cag) at position 80 has been changed to leucine (ctg) in the Q80L mutant and the two C-terminal cysteine residues (tgc tgc) have been deleted in the Δ C mutant.

tgc, corresponding to two C-terminal cysteine residues are deleted in this mutant in comparison to wild type sequence (Figure 16, Red). Previous results in higher eukaryotic cells demonstrated that C-terminal cysteine residues are required for prenylation, a post-translation modification, which subsequently aid Rab proteins to

attach with the membrane. Similarly, in the LdRab5:S34N mutant, the nucleotide sequence tcg was replaced by aac, which would substitute the serine (S) at position 34 by asparagine (N) (Figure 16, Blue). Same mutation in mammalian cells was shown to result in a GDP-locked negative mutant. The sequence of the LdRab5:Q80L mutant revealed that the nucleotide sequence cag, coding for glutamine (Q) was mutated to ctg, coding for leucine (L) (Figure 16, Green). After confirming the mutations, these products were sub-cloned into pGEX-4T-2 expression vector and recombinant mutant proteins were purified as GST fusion proteins. These mutant proteins were subsequently characterized to determine the role of *Leishmania* Rab5 in transport

3.3.7 Characterization of LdRab5 mutants

The mutant LdRab5 proteins were analyzed by SDS-PAGE. The profile revealed that all purified mutant fusion proteins typically migrated at ~50kDa, similar to GST-LdRab5:WT protein (Figure 17a). The lower molecular weight protein corresponding to ~25kDa possibly could be free GST. These results were further stre-

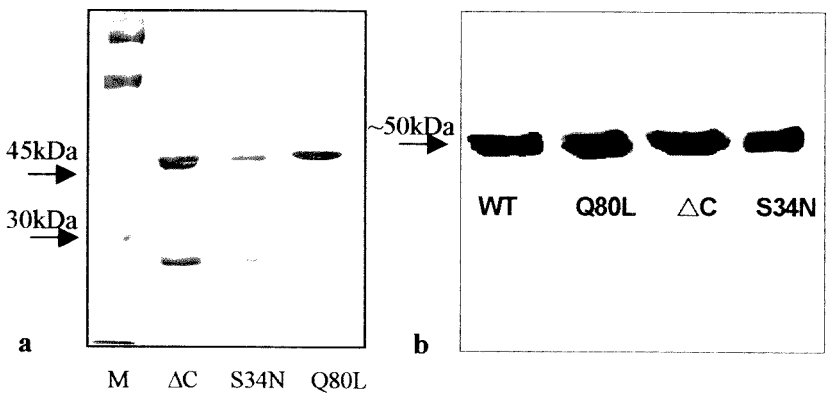


Figure 17. LdRab5 mutants were expressed as GST-fusion proteins and affinity purified with glutathione agarose beads and eluted with reduced glutathione. The purified proteins were analyzed by SDS-PAGE (**a**). M represents the molecular weight markers. Subsequently, respective mutant proteins were subjected to Western blotting using anti-LdRab5 antibody. The LdRab5:WT was used as a control (**b**).

-ngthened by Western blot analysis of mutant proteins using anti-LdRab5 antibody, which specifically detects ~50-kDa proteins in all the mutant samples. These results demonstrate that the purified proteins are *Leishmania* Rab5, containing the respective

mutations (Figure 17b). Subsequently, these mutant proteins were analyzed for their GTP binding ability and intrinsic GTPase activity.

The GTP binding assay revealed that LdRab5: Q80L and LdRab5: ΔC bind [α^{32}P]-GTP as efficiently as wild type protein whereas very reduced binding is observed with LdRab5: S34N (Figure 18). The observed reduced binding of [α^{32}P]-GTP by LdRab5:S34N could be because this mutant is locked in GDP form and unable to bind GTP, similar to the results shown in higher eukaryotic cells. No change in the nucleotide sequence was made in the GTP binding region of LdRab5:ΔC, accordingly, there was no alteration of [α^{32}P]-GTP binding ability of this mutant.

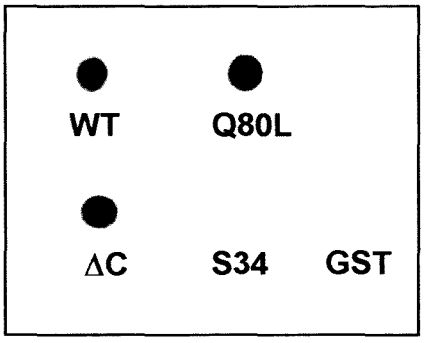


Figure 18. GTP binding analysis of different LdRab5 mutants. GTP binding for indicated mutants of LdRab5 was detected using [α-^{32}P] GTP overlay assay. Free GST were used as control.

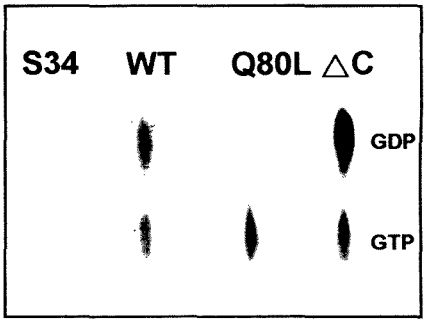


Figure 19. GTPase activity of LdRab5 along with the indicated mutants. GTPase activity was determined as stated in Materials and

Though LdRab5:Q80L was found to bind very efficiently with [α^{32}P]-GTP, however, the results presented in the Figure 19 show that this mutant is unable to hydrolyze bound GTP to GDP suggesting the reduced intrinsic GTPase activity of this mutant. No GTP hydrolysis was observed in case of LdRab5:S34N mutant owing to its defect in GTP binding. LdRab5:ΔC showed GTP hydrolyzing activity comparable to wild type protein. These results demonstrate that along with LdRab5, we have generated GTP and GDP-locked mutants of *Leishmania* Rab5, which will be useful in dissecting out the role of Rab5 in vesicular transport in this organism.

3.4 Discussion

Previous studies using cell free systems have shown that mammalian Rab5 regulates multiple events in the early steps of endocytosis in mammalian cells. (Nielsen *et al.*, 1999; Gorvel *et al.*, 1991; Bucci *et al.*, 1992). In order to clone Rab5 from *Leishmania* and subsequently study its role in intracellular trafficking in *Leishmania*, we identified a putative Rab5 like sequence in *L. major* genome database using *T. brucei* Rab5B as a query (Field *et al.*, 1998). The Rab5-like gene from *Leishmania* was subsequently cloned and expressed as GST-LdRab5 fusion recombinant protein. The protein sequence of LdRab5 has the general features of all Rab proteins such as (a) size of the protein ranges from 20-29-kDa; (b) conserved GTP and phosphate binding domains; (c) presence of C-terminal cysteines. It has been shown using Rab5 sequences from various sources that this protein has specific signature motifs like RYKS and YYRGA (Stenmark and Olkkonen, 2001; Pereira-Leal and Seabra, 2000). Our results show that these signature motifs are also present in the cloned *Leishmania* Rab5 protein. Moreover, BLAST analysis of the LdRab5 sequence reveals significant homology with Rab5 from different organisms. The predicted structure of LdRab5 based on the recently solved crystal structure of mouseRab5c (Merithew *et al.*, 2001), reveals the presence of six β-sheets, comprising five parallel strands and one antiparallel strand, surrounded by five α-helices. Taken together, these results suggest that the cloned protein from *Leishmania* is indubitably a Rab5 homologue.

Rab proteins are GTPases belonging to a large family with homology to Ras (Olkkonen and Stenmark, 1997). Several lines of evidence have demonstrated that Rab proteins regulate every intracellular transport step through a nucleotide-dependent conformational change (Zerial and McBride, 2001; Pfeffer, 2001). This regulation of intracellular transport is mediated through cyclical binding and hydrolysis of GTP. Thus, the GTP binding ability and intrinsic GTPase activity are key features of Rab proteins. The results (Figure 9) show that recombinant LdRab5 specifically binds [α^{32}P]-GTP and this binding is competed out by unlabeled GTP and GDP but not by ATP, thus confirming the specificity of LdRab5 for guanine nucleotides. Though, LdRab5 is able to bind significant amount of GTP, the rate of GTP hydrolysis of LdRab5 appears to be slower in comparison to the 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 (Olkkonen and Stenmark, 1997). 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.

GTP hydrolysis of Rab5 acts as a timer that determines the frequency of membrane docking/fusion events (Rybin *et al.*, 1996). Rab5 has one of the highest basal GTPase rates among members of the Rab family and regulates one of the fastest intracellular transport processes. According to Zerial and colleagues, "The requirement for such a dynamic GTPase cycle becomes apparent if one considers the dual role of Rab5 in the fusion of clathrin coated vesicles with early endosomes and the homotypic fusion between early endosomes". The difference in the GTP hydrolysis profile between the two proteins may be attributed to the phylogenetic differences between the two organisms. In addition to this, Rab5 is known to be a rate-limiting component of the early endocytic machinery (Bucci *et al.*, 1992). It will be interesting to study whether *Leishmania* Rab5 functions in a similar fashion. Though LdRab5 has about 65% similarity with human/mouse Rab5, the antibody raised against the *Leishmania* protein does not react with mouse protein or vice-versa as revealed by Western blot analysis (Figure 12a). These results reveal the species specificity of Rab5. Rab5 has been shown to be present in other protozoa including members of the family Trypansomatidae like *T.brucei* and *T.cruzi* where it has been shown to be associated with the endocytic compartments. The two isoforms of Rab5, TbRab5A and TbRab5B in *T. brucei*, are involved in distinct trafficking steps. TbRab5B is involved in trafficking of trans-membrane proteins like ISG100, whereas TbRab5A regulates endocytosis of GPI anchored proteins e.g. transferrin receptors (Pal *et al.*, 2002). The amino acid sequence reveals that LdRab5 shares homology with TbRab5B isoform suggesting a possible role for LdRab5 in the trafficking of transmembrane proteins in *Leishmania*. Since a large number of membrane proteins in trypanosomatids are GPI- anchored, it is likely that isoforms of Rab5, which may be involved in the trafficking of GPI-anchored proteins in *Leishmania,* may exist.

Rab proteins cycle between GTP and GDP bound confirmations, the GTP bound form being known as "on" switch for vesicular transport. By analogy with the known structure of Ras, the nucleotide bound state of Rab proteins is believed to dictate their conformation and molecular interactions (Jurnak *et al.*, 1990; Tong *et al.*,

1989). Previous studies have shown that when the nucleotide binding and hydrolysis cycle is disrupted by appropriate mutations in the highly conserved region of Ras related proteins, usually transport is altered or inhibited (Bucci *et al.*, 1992; Riederer *et al.*, 1994; Tisdale *et al.*, 1992). Accordingly, several mutants have been made by alterations in the conserved domains of these proteins in order to determine their role in transport. Earlier results have shown that substitution of leucine to glutamine (Q to L) in the WDTAGQE region of Rab proteins or exchange of asparagine for serine (S to N) generates proteins, which are constitutively locked in the GTP or GDP bound conformation, respectively (Frech *et al.*, 1994; Nuoffer *et al.*, 1994, Stenmark *et al.*, 1994, Tisdale *et al.*, 1992). Most of the Rab5 interacting proteins, which regulate intracellular transport, have been identified using similar mutant proteins. Thus, attempts have been made in the present study to generate analogous mutants of LdRab5.

The Rab5:Q79L mutation in mammalian Rab5 results in the protein having greatly reduced GTP hydrolysis activity, making it constitutively active. The analogous mutant, LdRab5:Q80L in *Leishmania* also exhibits a reduced GTP hydrolysis activity. In contrast, Rab5:S34N is shown to be a dominant negative mutant and inhibits fusion between early endosomes (Gorvel *et al.*, 1991). Similarly, we have prepared LdRab5:S34N mutant, which is locked in GDP form. It has been also shown that deletion of cysteine residues from C-terminus of Rab proteins that are required for prenylation, results in the failure of delivery of the protein to the membrane and inhibits transport. We have made analogous ΔC mutant from *Leishmania* in the present study. Further studies using these mutant proteins will be useful to determine the regulation of endocytosis in *Leishmania.*

Chapter 2

Role of LdRab5 in fusion between early endosomes in Leishmania promastigotes

4.1 Introduction

Rab proteins are the key regulators of vesicular transport both in endocytic and secretory pathways. Rab proteins localize to the cytoplasmic surface of intracellular compartments and control membrane trafficking at specific steps. For example, Rab4, Rab5 and Rab11 are localized to early endosomes and serve different functions. Rab4 is shown to be involved in the regulation of recycling of transferrin from the early endosome (van der Sluijs *et al.*, 1991). Rab5 mediates the homotypic fusion between early endosome as well as in the transport from plasma membrane to the early compartments (Gorvel *et al.*, 1991). Rab11 on the other hand has been demonstrated to function in the transport through the recycling compartment (Wilcke *et al.*, 2000; Ullrich *et al.*, 1996). Rab7 and Rab9 are localized on the late endosomal compartments (Chavrier *et al.*, 1990; Lombardi *et al.*, 1993). Rab7 has been shown to control the late endocytic trafficking (Meresse *et al.*, 1995; Feng *et al.*, 1995; Vitelli *et al.*, 1997) while Rab9 regulates transport between late endosomes and trans-Golgi network (Lombardi *et al.*, 1993).

To determine the role of individual Rab proteins in membrane fusion, it is necessary to isolate and purify the vesicular compartments. Subcellular fractionation of the cell lysate by sucrose or percoll gradients has been employed successfully for purification of endocytic vesicles (Storrie *et al.*, 1984; Gorvel *et al.*, 1991). These studies have shown that early endosomes are lighter in density as compared to the late endocytic compartments, which are in turn lighter than the lysosomes (Helenius *et al.*, 1983). These fractions are further characterized by the presence of specific biochemical markers associated with purified vesicles, for example, early endosomes predominantly contain transferrin receptor; late endosomes and lysosomes are enriched in mannose-6-phosphate receptor and lysosomal glycoprotein1, respectively (Kornfeld and Mellman, 1989). Characterization of endocytic compartments has also indicated that Rab5 localizes to the lighter early endosomes while the denser late endosomes/lysosomes are enriched with Rab7 (Gorvel *et al.*, 1991).

Since, these endocytic compartments are located mostly on the cytoplasmic side of the membrane, they are largely inaccessible for direct biochemical manipulation to determine the role of particular Rabs and other signal transduction intermediates in membrane trafficking events. The classical biochemical approach to unravel cellular mechanisms involved in endocytosis is to develop a cell free assay to

measure fusion between two distinct populations of endocytic vesicles containing appropriate probes, in the presence of cytosol, which contains all necessary signaling molecules to provide the microenvironment necessary for their interactions (Gruenberg *et al.*, 1989). These *in vitro* assays have provided important insights into the mechanisms and regulation of intracellular trafficking of endocytosed molecules in higher eukaryotic cells (Balch, 1989). These studies have shown that homotypic fusion between two endosomes is regulated by Rab5 and transport from early compartment to late/lysosomal compartment is mediated by Rab7 (Gruenberg and Howell, 1986). Moreover, several Rab-associated factors needed for vesicle fusion have also been identified using similar reconstitution assays.

The present study involves the purification and characterization of Hb containing endosomes from *Leishmania* and the development of an *in vitro* reconstitution assay to determine the role of Rab5 in Hb trafficking in *Leishmania*.

4.2 Materials and Methods

4.2.1 Materials

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co (St. Louis, MO). N-hydroxy succicinimidobiotin (NHS-Bio), avidin-horseradish peroxidase (AHRP), avidin, were purchased from Vector laboratories, Inc (Burlingame CA) Bicinchoninic acid (BCA) reagent was purchased from Pierce Biochemicals, Rockford, IL. Lysotracker red was purchased from Molecular Probes, Eugene, USA. Other reagents used were of analytical grade. Recombinant GDI fusion construct was kindly provided by Dr. Philip Stahl (Washington University School of Medicine, St Louis, MO). Protein A/G agarose beads were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

4.2.2 Antibodies

Anti-Hb, anti- NSF, anti-Rab7 and anti-Rab5 antibodies were raised in mice by standard techniques (Overkamp *et al.*, 1988). Anti- transferrin receptor antibodies were purchased from Zymed Laboratories. Alexa Fluor 488 goat anti-mouse IgG was purchased from Molecular Probes, Eugene, USA. All the second antibodies labeled with HRP were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

4.2.3 Biotinylation of Hemoglobin

Hemoglobin (Hb) was biotinylated using NHS-biotin as described (Gruenberg *et al.*, 1989). Briefly, 20 mg of Hb was dissolved in 9.5 ml of 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.0 and 11.5 mg of NHS-biotin was added to the Hb solution. Mixture was incubated for 2 hr at room temperature with stirring and unreacted active groups were quenched by the addition of 1 ml 0.2 M glycine. Finally, biotinylated Hb was separated from the other small molecules by extensive dialysis against phosphate buffer saline, pH 7.2. The extent of biotinylation of Hb was checked by ELISA. Briefly, the ELISA plate was coated with biotinylated protein for 1 hr at 37°C. The plate was washed five times with PBS containing 0.1% Tween-20 and blocked with 2% BSA. Subsequently, AHRP (100ng/ml) was added and the plate was incubated at 37°C. Finally, the HRP activity associated with anti BHb-AHRP complex was measured to determine the presence of biotin attached with Hb using O-phenylenediamine as the chromogenic substrate. Unlabeled Hb was used as a control.

4.2.4 Preparation of early endosomes from *Leishmania*

To prepare early endosomes, *Leishmania* promastigotes were incubated with biotinylated hemoglobin (BHb) (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 BHb was stopped by the addition of cold medium and the cells were washed five times with PBS. Subsequently, 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 at 4°C. The cells were disrupted by release of N_2 from the bomb. 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) containing BHb-loaded early endosomes was quickly frozen in liquid nitrogen. Early endosomes containing AHRP were prepared under identical conditions.

4.2.5 Subcellular fractionation of *Leishmania donovani* endocytic compartments

To characterize the compartments labeled with 5 min internalization of AHRP at 23°C by *L. donovani* promastigotes, subcellular fractionation was carried out using a procedure similar to the one 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 a MLS 50 rotor (Beckman TL100) at 100,000g for 1hr at 4^0C, 50 µl fractions were collected from the top of the gradient and samples were analyzed for the presence of HRP activity and various biochemical markers namely Rab5, Rab7 and transferrin receptor by Western blot analysis. The fractions were also analyzed for the plasma membrane marker, 5' nucleotidase activity. Similar fractionation was carried out using PNS prepared following 5 mins uptake of BHb and the presence of Hb in the fractions was detected by Western blot analysis using anti-Hb antibody.

4.2.6 *In vitro* reconstitution of endosome fusion in *Leishmania*

Reconstitution of fusion between endosomes prepared from *Leishmania* was carried out using a similar procedure described earlier for other systems (Gorvel *et al.*, 1991). Briefly, two sets of endosomes containing either BHb or AHRP were mixed 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, and 0.25 mg/ml avidin as scavenger; including an ATP regenerating system, 1 mM ATP, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase) 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). Finally, the BHb-AHRP complexes were immunoprecipitated using anti-Hb antibody and the HRP activity associated with BHb-AHRP complex was measured as fusion unit using O-phenylenediamine as the chromogenic substrate (Gruenberg *et al.*, 1989). Fusion carried out at 4°C or without cytosol was low and was subtracted from the other values to determine specific fusion.

4.2.7 Treatment of endosomes with GDI

In order to determine the role of Rab proteins in endosome fusion, the BHb-loaded endosomes were treated with Rab GDP dissociation inhibitor (GDI) as described previously (Garret *et al.,* 1994). The endosomes were preincubated with fusion buffer containing protease inhibitors (1mM phenyl methylsulfonyl fluoride, 20ug/mlleupeptin and 20ug/ml of aprotinin) for 20 mins at 23°C in the presence of 1mM GDP. Subsequently, 6 μg/ml of mammalian GDI was added to the reaction mixture and incubated for 20 mins at 23°C. The endosomes were sedimented by centrifugation at 100,000g for 5 mins and the pellet was assayed for the presence of Rab proteins by Western blot analysis using anti-LdRab5 antibodies. Presence of transferrin receptor was analyzed using appropriate antibody to confirm that GDI specifically retrieves Rab proteins from the membrane. The pellet, thus confirmed to contain Rab-stripped endosomes was washed with HB and used in *in vitro* fusion assay.

4.2.8 Immunodepletion of Rab5 from *Leishmania* cytosol

To deplete Rab5 from the cytosol, first, 100 μl of protein A/G plus–agarose beads were incubated with 10 μl of anti-LdRab5 antibody in PBS overnight at 4°C. The beads were then washed thrice with PBS and blocked with 1mg/ml of BSA in PBS for 1hr at 4°C. Subsequently, 600 μg of *Leishmania* cytosol containing protease inhibitors was added to the protein A/G agarose-anti-LdRab5 complex and incubated for 1hr at 4°C to deplete Rab5 from the cytosol as described previously (Mukherjee *et al.*, 2001). Subsequently, Rab5-depleted cytosol was separated from the agarose beads by centrifugation. Similar procedure was used to deplete the NSF-like protein as well as Rab7 from the *Leishmania* cytosol using anti-mammalian NSF and anti-LdRab7 antibodies, respectively.

4.2.9 *In vitro* prenylation of recombinant Rab proteins

The post-translational modification of Rab proteins i.e., the attachment of isoprenoid moiety to the C-terminal cysteine residues is a pre-requisite for their function. Rab proteins attach to the membranes of specific compartments through this lipid moiety. However, recombinant proteins expressed in *E.coli* do not undergo post-translational modifications. Thus, recombinant LdRab5 was incubated with

Leishmania cytosol immunodepleted of Rab5 for 30 mins at 23°C, to allow for *in vitro* prenylation. Subsequently, this cytosol was used in the fusion assay.

4.2.10 Immunolocalization of Rab5 in *Leishmania donovani*

Early endosomes were labeled by 5 min uptake of BHb at 23°C and lysosome -like compartments were labeled by incubating the promastigotes with Lysostracker Red (10μM) (Molecular probes) for 30 min at 23°C in PBS before fixing the cells. Cells were washed thrice with PBS and fixed in 4% formaldehyde in PBS for 20 min at 4 °C. Cells were washed and thin smears were prepared on glass slides and permeabilized with 0.4% saponin for 20 min at room temperature. In order to localize Rab5 in *L. donovani* promastigotes, cells were incubated with 10% FCS to block nonspecific binding and were probed with mouse anti-LdRab5 antibody in PBS containing 0.4% saponin for 30 min at room temperature. Cells were washed thrice with PBS and incubated with Alexa Fluor 488 labeled goat anti-mouse in the same buffer for 30 min to allow the detection of primary antibody binding. Early endosomes labeled with BHb were visualized by avidin-Texas Red. Finally, the cells were washed and incubated for 15 min with 5μg/ml of propidium iodide to label the nucleus and kinetoplast. The slides were then mounted with antifade reagents (Molecular Probes) and viewed in a LSM 510 confocal microscope using oil immersion objective.

4.3 Results

4.3.1 Subcellular fractionation of *Leishmania donovani* endocytic compartments

Previously it has been shown that initial binding of Hb with hemoglobin receptor leads to the efficient internalization of Hb within 5 min into endocytic compartment in *Leishmania* (Sengupta *et al.*, 1999). Thus, 5 min internalization of the BHb was used to label the endocytic compartment in *Leishmania* through receptor mediated uptake process. Another set of endosomes was labeled with 5 min internalization of AHRP as a fluid phase marker. In order to characterize compartment labeled with these probes, subcellular fractionation was carried out as described in the experimental procedure and HRP was estimated from different

fractions (50 µl) collected from the top of the gradient. Maximum HRP activity was obtained at 8-30% interface (Figure 20). 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.

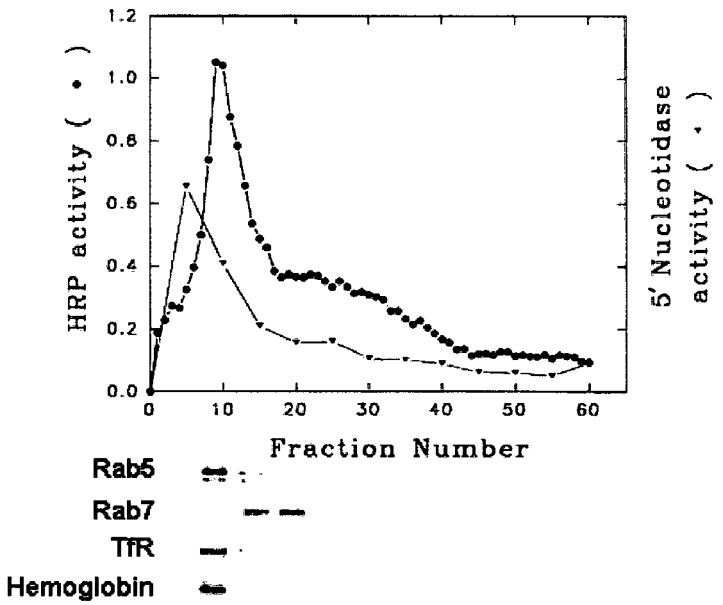


Figure 20. Subcellular fractionation of *L. donovani* endocytic compartment. Promastigotes were disrupted after internalization of AHRP and PNS was loaded onto a discontinuous sucrose gradient as described in Materials and Methods. After centrifugation, 50 µl fractions were collected from the top of 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 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.

The results presented in the Figure 20 show that Rab5 is predominantly present in fraction numbers 6-10, which retain maximum HRP activity, whereas, Rab7 positive compartment is found in the later fractions with relatively low HRP activity. Similarly, transferrin receptor was seen to be present in the fractions, which retained maximum HRP activity and were positive for Rab5. 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 (Figure 20). The fractions were also tested for the presence of plasma membrane enzyme, 5' nucleotidase and maximum activity of this enzyme was detected in the lighter fractions having relatively lower HRP activity.

4.3.2 *In vitro* reconstitution of homotypic early endosome fusion

Reconstitution of endosome fusion has been successfully used to determine the requirements for endocytosis (Gorvel *et al.*, 1991). The results presented in Figure 21 show a typical *in vitro* fusion experiment in which two sets of endosomes containing BHb or AHRP were incubated for 1 hr at 23°C in the presence of ATP regenerating system containing different concentrations of gel filtered cytosol prepared from *Leishmania* promastigotes. Maximum fusion between the endosomes was observed at cytosol concentration of 1mg/ml. No endosome fusion was detected in the absence of cytosol, suggesting the role of cytosolic components in endosome fusion. To determine energy requirements, endosome fusion was carried out in ATP

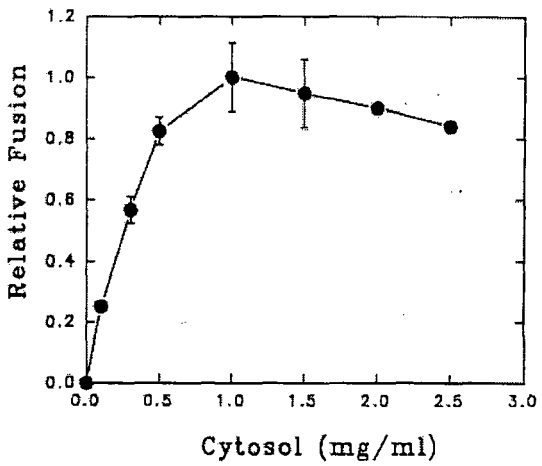


Figure 21. Cytosol dependent fusion o homotypic early endosomes using *in vitr* reconstitution assay. Two sets of earl endosomes containing BHb or AHR were incubated in the presence of AT regenerating fusion buffer supplemente with different concentrations of ge filtered *Leishmania* cytosol for 1 h a 23°C. Maximum fusion was observed at mg/ml of cytosol, which was normalize to 1 unit and results are expressed a relative fusion of three independen experiments ± S.D. One unit correspond to 12 ng of HRP activity/mg of protein.

depleting system (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2, 1 mM dithiothrietol, 1.5 mM MgCl2, 100 mM KCl containing 5mM glucose and 25 units/ml hexokinase and 0.25 mg/ml avidin as scavenger). Under these conditions, as well as in the presence of ATP regenerating system containing 4.5 μM ATPγS, significant inhibition of fusion was observed between endosomes, indicating that both ATP and ATP hydrolysis are required for this process (Figure 22). Moreover, fusion

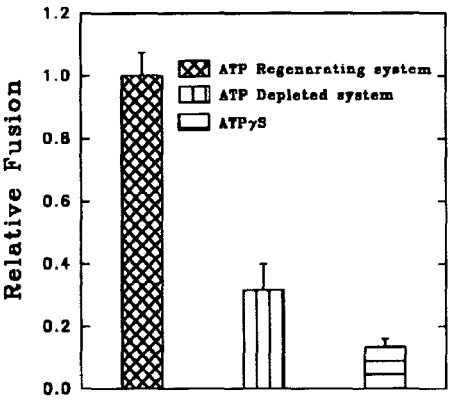


Figure 22. Role of ATP in the fusion between early endosomes. *In vitro* fusion between endosomes from *Leishmania* was carried out in ATP regenerating system (Control), ATP depleted system or in the presence of ATPγS (4.5 μM). Fusion obtained in control was chosen as 1 unit and the results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 13.6 ng of HRP activity/mg of protein.

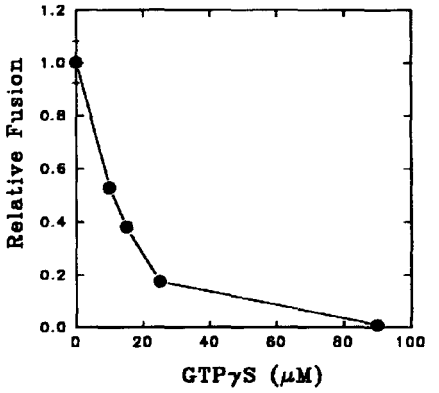


Figure 23. Effect of GTPγS on early endosome fusion. Fusion between endosomes in *Leishmania* was carried out in ATP regenerating system in the presence of different concentrations of GTPγS and 1.5 mg/ml cytosol. Fusion obtained in the absence of GTPγS was chosen as 1 unit and the results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 12.6 ng of HRP activity/mg of protein

between early endosomes at a high cytosol concentration (1.5 mg/ml) was inhibited by 25μM GTPγ S, a non-hydrolysable analog of GTP by about 80% (Figure 23). These results suggest that one or more GTPases regulate the *in vitro* homotypic fusion of early endosomes.

To determine the role of Rab GTPases in this process, endosomes were treated with GDI (GDP dissociation inhibitor) in the presence GDP, which was shown to specifically remove Rab protein from the membrane. The results presented in Figure 24 show that mammalian GDI the presence of 1 mM GDP specifically removed *Leishmania* Rab5, but not transferrin receptor from the endosomal membrane. Subsequently, these Rab-stripped endosomes were used in the *in vitro* fusion assay, wherein fusion was inhibited to about 90% in comparison to control (Figure 25). However, GDI alone did not affect the efficiency

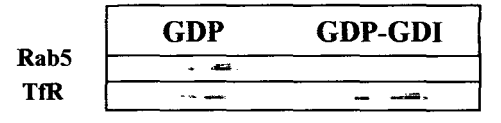


Figure 24. Endosomes containing BHb treated either with GDP (1mM) alone or with GDI (6μg/ ml) as described (Mukherjee *et. al.*, 2000), were centrifuged and pellets were analyzed for the presence of Rab protein by Western blot using anti-LdRab5. Transferrin receptor (TfR) was used as a control.

of homotypic early endosome fusion. These results strongly suggest the role of Rab-like GTPases in early endosome fusion in *Leishmania.*

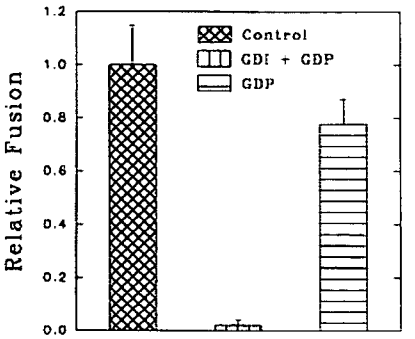


Figure 25 Early endosomes treated with GDP alone or GDP-GDI were used in the fusion assay. Fusion obtained with untreated endosomes was chosen as 1 unit and results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 14.5 ng of HRP activity/mg of protein.

4.3.3 Role of NSF-like protein in endosome fusion in *Leishmania*

Earlier studies demonstrated that *in vitro* fusion between endosomes from mammalian cells is inhibited by NEM treatment and can be restored by addition of NEM-sensitive factor, NSF (Diaz *et al.*, 1989). To detect the role of NSF-like protein in early endosome fusion in *Leishmania*, fusion was carried out in the presence of different concentrations of NEM. Figure 26a shows that about 70 % of the fusion was

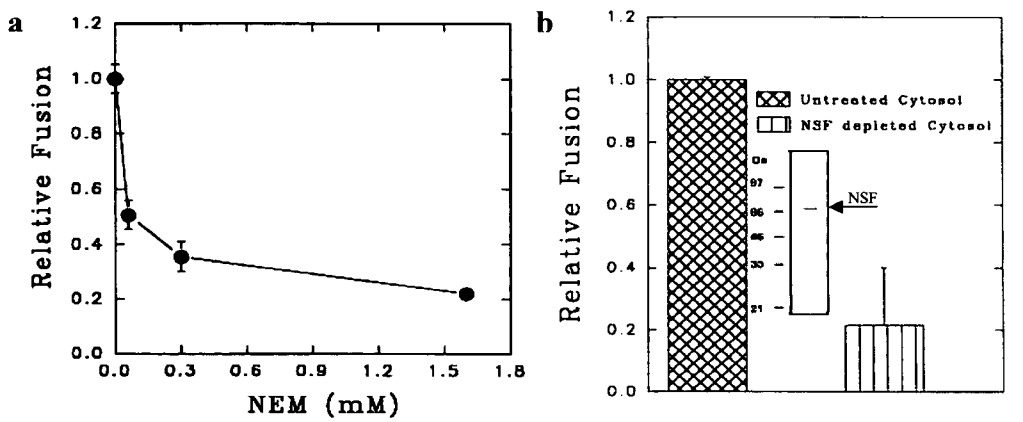


Figure 26. Role of NSF-like protein in endosome fusion in *Leishmania.* (**a**) Cytosol prepared from *Leishmania* was treated with different concentrations of NEM (30 min at 4°C) and excess NEM was quenched with 3mM dithiothreitol. Fusion was carried out in ATP regenerating system containing NEM-treated cytosol. Fusion obtained in the absence of NEM was chosen as 1 unit and the results are expressed as relative fusion of three independent experiments ± S.D. 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 unit and the results are expressed as relative fusion of three independent experiments ± S.D. 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.

inhibited by 0.3 mM NEM. Moreover, anti-mammalian NSF antibody specifically recognized a ~70kDa protein in *Leishmania* lysate, which could possibly be an NSF homologue in *Leishmania* (Figure 26b). Thus, NSF-like protein was immunodepleted from *Leishmania* cytosol using this antibody. Fusion between early endosomes in presence of NSF immunodepleted cytosol was significantly inhibited with respect to control cytosol, demonstrating the role of NSF-like protein in endosome fusion in *Leishmania* (Figure 26b).

4.3.4 Role of endocytic Rabs in intracellular trafficking of Hb:

To determine the role of endocytic Rabs in endosome fusion in *Leishmania*, cytosol was treated with anti-LdRab5 or anti-LdRab7 antibodies. The depletion of respective proteins from the cytosol was confirmed by Western blotting (Figure 27), which demonstrated that Rab5 and Rab7 could be specifically immunodepleted from *Leishmania* cytosol by specific antibody. Subsequently, these immunodepleted cytosol were used in the fusion assay to determine the role of Rab5 and Rab7 in early endosome fusion in *Leishmania*. Fusion between early endosomes was inhibited by about 75% in Rab5 depleted cytosol, whereas Rab7 depletion reduced fusion by only about 25% (Figure 28). Addition of *in vitro* prenylated LdRab5 (Lombardi *et al.*, 1993) to the Rab5 depleted system completely restored fusion to the control level (Figure 28).

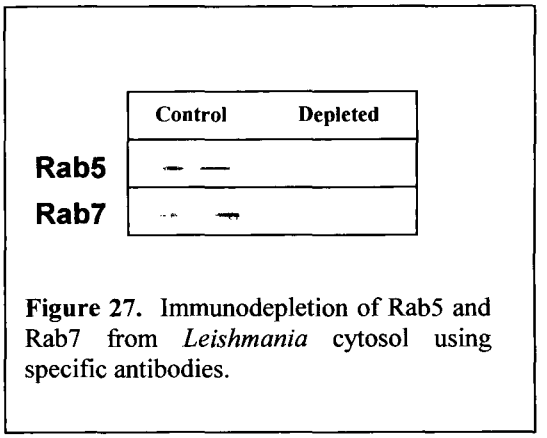


Figure 27. Immunodepletion of Rab5 and Rab7 from *Leishmania* cytosol using specific antibodies.

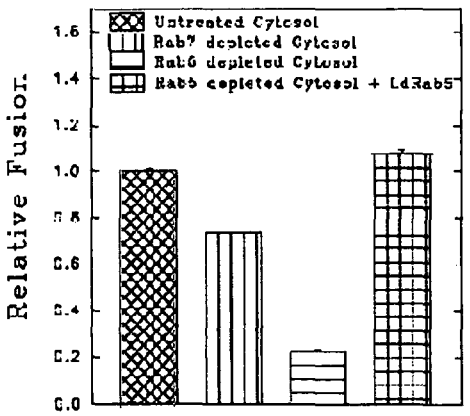


Figure 28. Role of endocytic Rabs in endosome fusion in *Leishmania*. Fusion between early endosomes were carried out in the presence of untreated cytosol, LdRab7 depleted cytosol, LdRab5 depleted cytosol or LdRab5 depleted cytosol supplemented with 500 ng of *in vitro* prenylated LdRab5. Fusion obtained with untreated cytosol was chosen as 1 unit and the results are expressed as relative fusion of three independent experiments ± S.D. One unit corresponds to 13.2 ng of HRP activity/mg of protein

4.3.5 Immunolocalization of Rab5 in *Leishmania donovani*

To determine the localization of Rab5 in *Leishmania* promastigotes, permeabilized cells were probed with anti-LdRab5 antibody followed by second antibody labeled with fluorescent dye. First, the plasma membrane and nucleic acids in *Leishmania* promastigotes were labeled with lipophilic dye FM 4-64 and vital dye Syto-green, respectively. The results presented in the Figure 29 show that *Leishmania* has two nuclear bodies, the nucleus and the kinetoplast. The nucleus is located in the

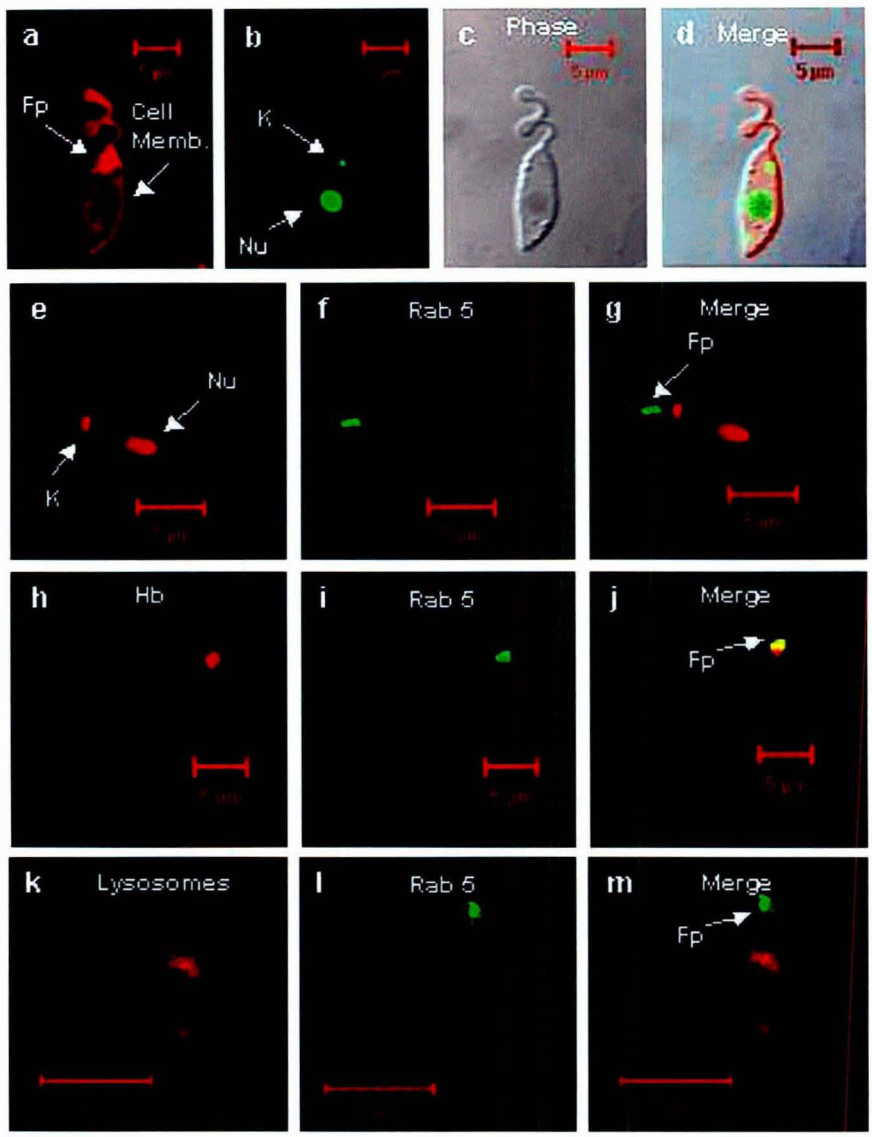


Figure 29. Confocal images showing the localization of Rab5 in *L. donovani* promastigotes. Rab5 was visualized by anti-LdRab5 antibody (**f, g, i, j, l, m**) and early endosomes were visualized by 5 min uptake of BHb (**h, j**). Yellow indicates colocalization of Rab5 with Hb in early endosomes (**j**). Nucleus (Nu) and kinetoplast (K) were labeled with propidium iodide (**e, g**) and lysosome-like compartments were indicated by lysotracker Red (**k, m**). Upper panel shows the phase image (**c**) of plasma membrane labeled with FM4-64 (**a**) and kinetoplast and nucleus stained with Syto green (**b, d**). Fp indicates the flagellar pocket.

center of the cell, while the kinetoplast is located at the base of the flagellar pocket from where flagella originates (panel a-d). Our results also demonstrate that Rab5 localizes above the kinetoplast, near the flagellar pocket (e-g). Moreover, Rab5 was found to co-localize with 5 min internalized Hb suggesting that Rab5 is located in the early endosomal compartment in *Leishmania* promastigotes (j). However, Rab5 positive early compartments were well separated from Lysotracker Red labeled lysosome-like acidic compartments, further strengthening the fact that Rab5 localizes to early endosomal compartments.

4.4 Discussion

Rab proteins are localized on distinct intracellular compartments and regulate specific transport steps, thereby serving as excellent markers to identify particular compartments. In the endocytic pathway, distinct Rab proteins localize to the early and late compartments, which facilitates identification of these compartments. Previous studies in different systems have shown that endocytic probes internalized for 5 min specifically reside in Rab5 positive early endosomes (Gorvel *et al.*, 1991), while probes internalized for 20-30 min predominantly label Rab7 positive late endosomal compartments. Further, early endosomes which are tubulovesicular structures can be morphologically differentiated from late endosomes which are multivesicular bodies (Geuze *et al.*, 1983, Geuze *et al.*, 1989). Moreover, early endosomes are shown to have a lesser density than late endocytic compartments (Storrie *et al.*, 1984; Wall and Hubbard, 1985). Thus, a commonly used criterion to separate and purify these compartments is density of the compartment labeled with appropriately internalized endocytic probes. The nature of early and late endosomes is further characterized by the presence of compartment specific markers like transferrin receptor, M6PR etc. Transferrin receptor is shown to recycle back to the plasma membrane from the early endosomal compartments, thereby servings as a specific marker for the early endosomes (Omary and Trowbridge, 1981; Mayor *et al.*, 1993). Similarly, CD-M6PR (M_r 46 kDa) and CI-M6PR (M_r 215 kDa) involved in the transport of lysosomal enzymes and predominantly present on late endosomal/prelysosomal compartments (Kornfeld, 1992; Stein *et al.*, 1987; Rabinowitz *et al.*, 1992; Griffiths *et al.*, 1990) serve as specific markers for late endosomes.

Based on our previous findings (Sengupta *et al.*, 1999), early endocytic compartments in *Leishmania* were labeled by 5 min internalization of BHb or AHRP. Cell fractionation data presented in Figure 20 shows that after 5 min internalization, the endocytic probes, AHRP as well as BHb are located in the fractions, which are enriched in Rab5. Similarly, transferrin receptor, which is a marker for the early compartments, is also present in the compartment labeled by 5 min internalization of AHRP or BHb. These results suggest that these compartments in *Leishmania* are early endocytic compartments. In contrast to mammalian cells, recent studies have shown that transferrin receptor in *Trypanosoma* is targeted to the lysosomal compartment (Steverding *et al.*, 1995). Interestingly, fractionation data have shown that in *Leishmania*, a trypanosomatid parasite, transferrin receptor is present only in the early compartment as is the case with mammalian cells.

In *Leishmania,* endocytosis takes place exclusively through the flagellar pocket (Overath *et al.*, 1997) and therefore, it is expected that the early endosomal compartment will be located near the site of endocytosis in *Leishmania* in correlation with the mammalian system where the early endosomes are present in the periphery just beneath the plasma membrane. The subcellular fractionation and localization studies in the present investigation have shown that 5 min internalized Hb colocalizes with Rab5 labeled compartments in *Leishmania* and these early endocytic compartments are located near the flagellar pocket. Previous studies from our laboratory have demonstrated that a putative hemoglobin receptor is located in the flagellar pocket of *Leishmania*. Thus, binding of Hb in the flagellar pocket, followed by short internalization correlates well with the localization of Hb in the early endosomes in the flagellar reservoir. Recently, it has been shown that Rab7 is localized in perinuclear late endocytic compartment in *Leishmania major* (Denny *et al.*, 2002). Our results show that during 5 min internalization, Hb is not transported to late/lysosomal compartment as revealed by Lysotracker Red staining and cell fractionation studies.

To understand the intracellular trafficking of Hb in *L. donovani* promastigotes and it's sorting in the early endocytic compartments, 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. 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). Thus, early endosome fusion in *Leishmania* requires cytosol, ATP and its hydrolysis (Diaz *et al.*, 1989; Gorvel *et al.*, 1991). Significantly higher level of fusion between endosomes from *Leishmania* was observed at its optimal growth temperature of 23°C rather than at 37°C, the optimal temperature for growth of mammalian cells. This is not surprising since *Dictyostelium*, which grows optimally at 21-28°C exhibits significant fusion at similar temperatures (Lenhard *et al.*, 1992). It has been shown in several systems that Rab-GDI in presence of GDP specifically depletes Rab proteins from the membrane (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). 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 (Weidenhaupt *et al.*, 1998; Kissmehl *et al.*, 2002). Accordingly, in our study, anti-mammalian NSF antibody specifically recognized a ~70 kDa protein in *Leishmania* and fusion carried out in presence of cytosol immunodepleted using this antibody was significantly inhibited, demonstrating the role of NSF-like protein in endosome fusion in *Leishmania*. These results along with others (Chaturvedi *et al.*, 1999; Bogdanovic *et al.*, 2000) suggest that, as in higher eukaryotic cells, an NSF-mediated SNARE complex is likely to regulate endocytosis in *Leishmania*.

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.

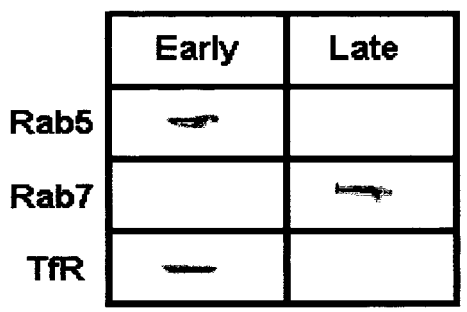


Figure 30. AHRP labeled early and late endosomes purified by sucrose density gradient were analyzed for the presence of early and late compartment-specific markers by Western blot using indicated antibodies (TfR, transferrin receptor).

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-

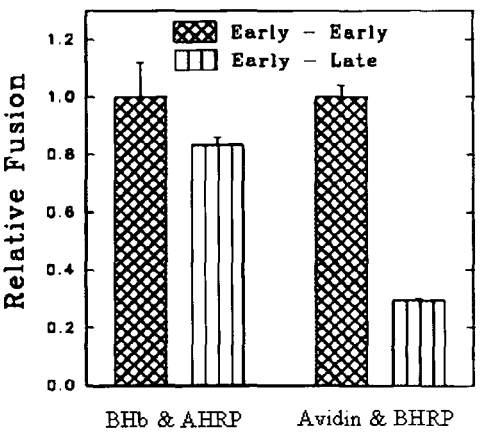


Figure 31. Fusion of early endosomes containing BHb with early or late endosomes containing AHRP were carried out in ATP regenerating system in the presence of cytosol. Similarly, fusion of early endosomes containing fluid phase marker like avidin was carried out with early or late endosomes containing 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

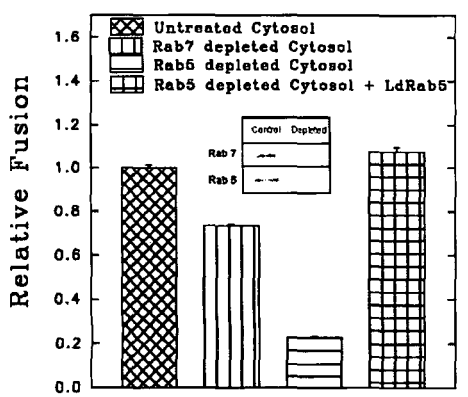


Figure 32: Heterotypic fusion between BHb-loaded early endosomes and late endosomes containing AHRP was carried out in ATP regenerating system in the presence of untreated cytosol, LdRab5 depleted cytosol, LdRab7 depleted cytosol or LdRab7 depleted cytosol supplemented with 500 ng of *in vitro* prenylated 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

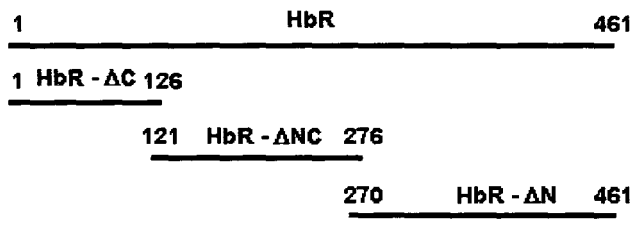


Figure 33. Deletion mutants HbR-ΔC, HbR-ΔNC and HbR-ΔN, corresponding to the N-terminus, middle region and C-terminus of HbR, respectively, were expressed and purified as GST fusion proteins.

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

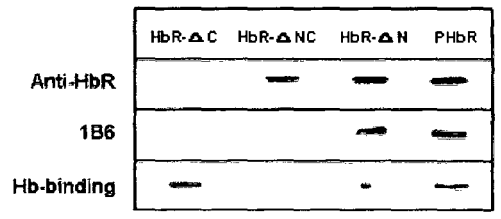


Figure 34. Purified HbR and deletion mutants (1μg/ lane) were analyzed by Western blot using anti-HbR polyclonal antibody (Top), monoclonal antibody, 1B6 (Middle) and their ability to bind hemoglobin (Bottom).

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.

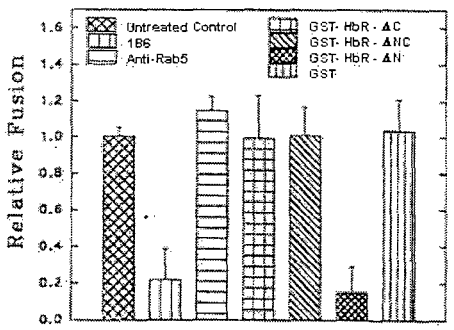


Figure 35. To determine the role of Hb cytoplasmic domain, BHb containing earl endosomes were incubated with 1B6, anti-LdRab antibody or indicated deletion mutants of HbR (28 ng) for 30 min at 4°C. Subsequently, late endosomes containing AHRP were added into treated earl endosomes and fusion was determined. 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.

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.

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