The *Leishmania donovani* peroxin 14 N-terminal region is important for glycosomal localization

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Abstract

Glycosomes are subcellular organelles that are evolutionarily related to the peroxisomes of higher eukaryotes. The *Leishmania* glycosome performs various metabolic processes that are essential for the survival of these parasites, such as the glycolytic process. Proteins that are destined for import into the glycosome interact selectively with specific cytosolic receptors peroxin 5(PEX5) or PEX7. The PEX5-protein complex migrates toward the glycosomal membrane where it interacts with PEX14, a vital step for protein important into the glycosome.

This project investigated the interaction mechanism of *Leishmania donovani* PEX14 with the glycosomal membrane. The regions responsible for PEX14 interaction with the glycosomal membrane are established in higher eukaryotes. LdPEX14 is poorly conserved with respect to the other PEX14 homologues. In *Leishmania* the interaction of LdPEX14 with the glycsomal membrane has been shown to be unique in terms of its lack of insertion in the glycosomal membrane. Using LdPEX14 mutants it was determined that the first 63 amino acids are important for the interaction of LdPEX14 with the glycosomal membrane. Results further suggest that LdPEX14 is a homopolymer forming a complex of 20S in size which is vital for the proper functioning of the glycosome.

Résume

Les glycosomes sont des organelles subcellulaires reliés aux peroxysomes des eucaryotes supérieures. Les glycosomes de Leishmania sont impliqués dans d'important processus pour le parasite, notamment la voie glycolytique. Les protéines qui sont destinées au glycosome sont attachées sélectivement au Peroxine 5 (PEX5) ou PEX7, des récepteurs cytosoliques. Le complexe PEX5-protéine est importé à la membrane du glycosome où il interagi avec la protéine PEX14 attachée à la membrane.

Dans ce projet le mécanisme d'interaction de PEX14 de *Leishmania donovani* avec la membrane du glycosome a été examiné. Les régions responsables de l'interaction entre PEX14 et la membrane du glycosome ont été établies chez les eucaryotes supérieurs. LdPEX14 est peu conservé par rapport aux PEX14 des autres espèces, de plus LdPEX14 est une protéine attaché en périphérie de la membrane. L'interaction de LdPEX14 avec la membrane du glycosome semble être unique due à son absence d'insertion dans la membrane du glycosome. Avec des protéines mutantes de LdPEX14 nous avons déterminé que les premiers 63 acides aminés sont importants pour l'interaction avec la membrane du glycosome. Finalement, les résultats suggèrent que LdPEX14 est un homopolymère d'une taille de 20S qui est crucial pour le bon fonctionnement du glycosome.

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Contributions

The LdPEX14 mutant constructs were contributed by Kleber Madrid. This work was performed toward completion of his Ph. D. October 2005.

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I. INTRODUCTION

The trypanosomastid group of parasites includes *Leishmania* and *Trypanosoma.* These parasites contain a unique organelle referred to as a glycosome. It is a microbody organelle containing a single phospholipid bilayer that is evolutionarily related to the peroxisomes of yeasts and mammals (Opperdoes, 1987; Opperdoes and Borst, 1977). The glycosome compartmentalizes several important biological functions which include purine salvage, glycolysis, gluconeogenesis, pyrimidine and ether-lipid biosynthesis and β-oxidation of fatty acids (Opperdoes, 1987; Fairlamb, 1989; Rodriguez and Landfear, 2006). Unlike peroxisomes, the glycosomes of human pathogenic trypanosomes lack the enzyme catalase. However, a unique feature of the glycosome that reflects its application is the presence of the first seven enzymes responsible for glycolysis (Opperdoes *et al.* 1988).

In *Trypanosoma brucei,* the glycosome is essential for survival of the bloodstream form of the parasite within the host (Bakker *et al.* 2000; Blattner *et al.* 1998; Guerra-Giraldez *et al.* 2002). Therefore the study of glycosome biogenesis offers an important window into the possible exploitation of this organelle for the treatments of trypanosomastid diseases. It may be possible to disrupt protein import into the glycosome, preventing the biologically relevant functions from occurring thereby compromising the parasites survival.

Proteins that function within the glycosome are targeted to the glycosome via specific transport mechanisms. The import of proteins is essential due to the fact

that the glycosome contains no DNA or protein-synthesizing machinery. Therefore, proteins destined for the glycosome are translated by cytosolic ribosomes and then shipped post-translationally into the glycosome.

The import of proteins into the glycosomes requires a specific targeting sequence, referred to as Peroxisomal Targeting Sequence (PTSs). Two main PTS' have been described, PTS1 and PTS2. The specific signals for PTS1 import, are denoted by a C-terminal tripeptide involving the sequence Ala-Lys-Leu (AKL) or Ser-Lys-Leu (SKL) or variants thereof (Blattner *et al.* 1992; Lametschwndter *et al.* 1998). The PTS-2 signal is an N-terminal nonapeptide, R/K-L/I/V-X5-H/E-A/L, located within the first 20- 30 amino acids of the protein. A third targeting signal called PTS3 has been identified to be an internal signalling sequence, however its significance in terms of import is minimal. Proteins containing a PTS1 or PTS2 import signal are bound by the cytosolic receptor proteins peroxin 5 (PEX5) or peroxin 7 (PEX7), respectively (Subramani *et al.* 2000). Upon binding the cargo protein the resulting binary complex moves towards the glycosomal membrane where it binds to a glycosomal membrane docking complex containing a membrane associated protein, PEX14. Interaction of the PEX5-PTS1 or PEX7-PTS2 complex results in the import of the peroxisomal-targeted protein into the glycosome via an unknown mechanism.

It is well established that PEX14 is critical for glycosomal protein import (Furuya *et al.* 2002). The exact association of PEX14 with the membrane of the microbody appears to vary depending on the organism. In yeast and mammalian cells PEX14 is

an integral membrane protein, showing a hydrophobic transmembrane domain (Itoh and Fujiki, 2006). In *Leishmania*, extensive biochemical and immunocytochemical studies have demonstrated that PEX14 is a peripheral membrane protein that associates with the cytosolic face of the glycosomal membrane (Jardim *et al.* 2002). Further evidence regarding the *Leishmania* PEX14 protein peripheral membrane association is that this protein is readily expressed in large quantities in *E. coli* as a soluble protein. Triton X-114 phase separation experiments demonstrate that the LdPEX14 partitions exclusively in the aqueous phase consistent with a protein that does not insert into the lipid bilayer.

The objectives of this project will be to identify regions implicated in the interaction of LdPEX14 with the *Leishmania* glycosomal membrane. It will also be important to explore the implications of certain LdPEX14 mutants on the growth and health of the parasite.

II. LITERATURE REVIEW

A. Leishmaniasis

Leishmaniasis is considered one of the most important parasitic diseases in the world. According to the World Health Organization (Schallig and Oskam, 2002.) approximately 350 million people are at risk of contracting the disease in almost 88 different countries worldwide. There are four clinical manifestations of this disease that have been defined, muco-cutaneous, cutaneous leishmaniasis, diffuse-cutaneous visceral leishmaniasis. Cutaneous and muco-cutaneous leishmaniasis affect approximately 1.5 million people per year, and are characterized as lesions or nodules within the skin or the mucous membrane. The more severe form of the disease is visceral leishmaniasis, which can be deadly if left untreated. Visceral leishmaniasis affects a smaller number of individuals when compared to the two other forms (Schallig and Oskam, 2002), only 500, 000 cases per year. The manifestation of visceral leishmaniasis is substantially worse as the parasites targets to the internal organs most specifically the spleen, liver, bone marrow, and lymph nodes within cells of the macrophage lineage.

B. Life cycle

In leishmaniasis endemic regions, approximately 20 different species of *Leishmania* have been shown to cause the various forms of this disease. A large similarity of parasites classified in the genus *Leishmania* is that they are all obligate intracellular parasites (Alexander and Russell, 1992; Alexander *et al.* 1999). The *Leishmania* parasite contains two important stages, the sandfly stage as well as the human stage

(Fig. 1). In the vertebrate host, the parasites are exist as non-motile, obligate intracellular form known as an amastigote that are round in shape and non-flagellated (Alexander and Russell, 1992). In the insect vector, usually the female sandfly, parasites are classified as a promastigote, which appear long and slender and contain a flagellum. This form of the parasite is highly motile and survives in an extracellular environment in the midgut and salivary glands of the sandfly. The two differing forms of the parasite live in two very distinct hosts and hostile environments. To survive in their respective host, physiological changes are necessary on conversion from amastigote to promastigote (Sacks and Perkins, 1985). This transformation is accompanied by a remodeling of the parasite cell surface. Two important molecules develop on the surface of the promastigote, the first is the glycoconjugate, lipophosphoglycan (LPG) and the second is a zinc metalloprotease called GP63 (Davies *et al.* 1990). These are two examples of how the parasite must adapt in order for the promastigote form of the parasite to survive in the sandfly environment.

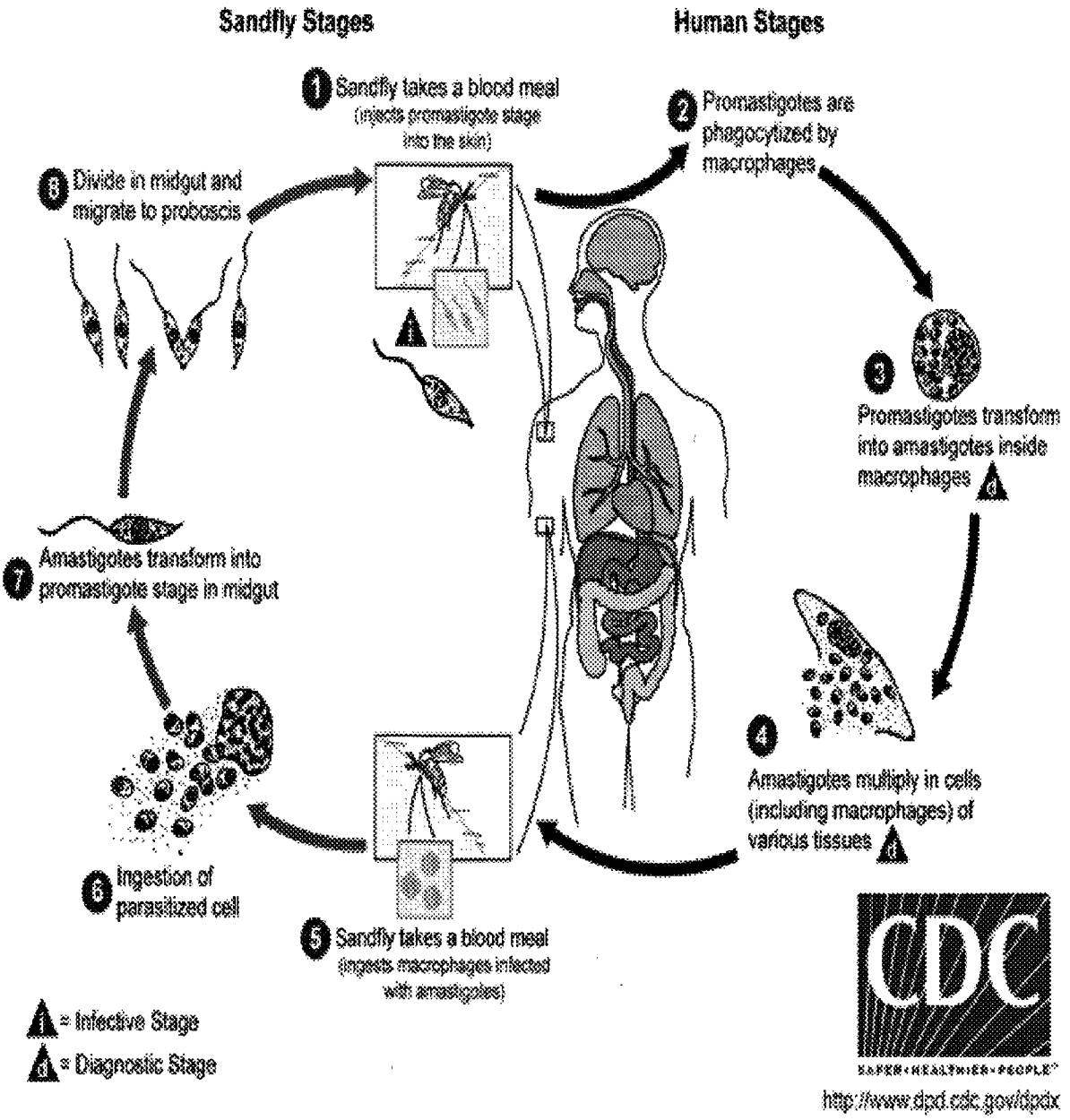

Figure 1. ***Leishmania donovani*** **life cycle.** 1) The sandfly vector takes a blood meal from a vertebrate host. 2) Promastigotes become phagocytized within the hosts macrophages. 3) Promastigotes differentiate into the amastigote form of the parasite. 4) Amastigotes multiply within the macrophage eventually leading to rupture of the cell. Amastigotes travel to other tissue sites of the host. 5) The sandfly takes another blood meal absorbing macrophages infected with amastigotes. 7) Promastigotes differentiate into the long slender promastigote form. 8) Promastigotes divide in midgut and migrate to proboscis. Cycle continues to other hosts. (CDC website)

C. Treatment

Vaccination would be a preferential means of controlling leishmaniasis, however there is currently no available vaccine against the forms of the disease. In contrast, a natural form of vaccination called leishmanization has been practiced for hundreds of years against the non-lethal cutaneous leishmaniasis were the disease is self-healing. For muco-cutaneous and visceral leishmaniasis the current form of chemotherapy most prevalently used for treating these infections has been pentavalent antimony (Sb (V)) (Hashim *et al.* 1995; Guerin, 2002). The mode of action of antimony therapy still remains largely unknown, however there is a general belief that to be active, Sb (V) needs to be reduced to the trivalent form (Ouellette and Ward, 2002). The presentation of increased drug resistance to the pentavalent antimonials in certain areas of Northeastern India have led to a requirement for new drug discoveries (Sundar, 2001).

Other chemotherapeutic agents used to treat leishmaniasis include the second-line anti-leishmanial drugs such as amphotericin B and more recently miltefosine. The limitations with respect to amphotericin B treatment include its toxicity and difficulty with administration (Olliaro and Bryceson, 1993). Miltefosine, an alkylphosphocholine, is a new orally available drug that is in clinical trials as a treatment for visceral leishmaniasis. Cure rates of 95% and 94% were observed after oral administration in phase II and phase III trials in Indian patients (Jah *et al.* 1999; Sundar *et al.* 2002). However, in laboratory studies, miltefosine-resistant clones of *L. donovani* have been readily isolated (Seifert *et al.* 2003), suggesting that

Leishmania possesses the mechanisms to develop resistance to this new drug. Moreover, this drug exhibits teratogenic effects which precludes its use in pregnant females. Therefore, a need persists for new chemotherapeutic agents against *Leishmania.* Unlike most other organelles, the glycosome import apparatus will accommodate fully folded, oligomeric and co-factor-bound substrates. Therefore it may be possible to directly target drugs to take advantage of this unique mechanism (Terlecky and Kopke, 2007).

D. Glycosomes: purpose and biogenesis

Within the vertebrate host the parasite must perform several functions in order to survive and propagate which include subversion of the host immune response and survival in stressful conditions with limited nutrients and energy sources. The glycosomes play an important role in parasite survival as the molecular processes that occur within the glycosome are its prime energy production mechanism, for instance glycolysis. This single membrane microbody organelle, although unique to the order Kinetoplastida, is evolutionarily related and shares a number of metabolic similarities with the peroxisomes of yeasts and mammals (Table 1.) (Opperdoes, 1987; Opperdoes and Borst, 1977). Glycosomes perform a variety of essential metabolic functions that include purine salvage, glycolysis, gluconeogenesis, pyrimidine and ether-lipid biosynthesis and β-oxidation of fatty acids (Opperdoes, 1987; Fairlamb, 1989; Rodriguez and Landfear, 2006). Moreover, the import of proteins into the glycosome has been discovered to be essential for the survival of *T. brucei* (Bakker *et al.* 2000; Blattner *et al.* 1998; Guerra-Giraldez *et al.* 2002).

Studies have demonstrated that the reduction in glycosomal protein import resulted in parasite death (Furuya *et al.* 2002). The import of peroxisomal proteins into the glycosome is required as no DNA is located within the glycosome. Proteins must be produced by ribosomes in the cytosol and then imported into the glycosome in order to perform their function (Sommer and Wang, 1994).

Trypanosomatids	Mammalian	Yeast
Ether-Lipid Biosynthesis (Opperdoes, 1984)	Ether-Lipid Biosynthesis	Ether-Lipid Biosynthesis
β-oxidation of fatty acids (Wiemer *et al.* 1996)	α- and β-oxidation of fatty acids	β-oxidation of fatty acids
Pentose phosphate pathway (Duffieux *et al.* 2000)	Pentose phosphate pathway (Wanders *et al.* 2003)	-----
Glycolysis	-----	-----
Gluconeogenesis (Hannaert *et al.* 2003)	-----	-----
Purine salvage (Zarella-Boitz *et al.* 2004)	-----	-----
Pyrimidine metabolism	-----	-----
-----	Reactive oxygen species metabolism (Catalase)	Reactive oxygen species metabolism (Catalase)
-----	Glyoxylate metabolism (Wanders *et al.* 2003)	-----
-----	Cholesterol synthesis (Wanders *et al.* 2003)	-----
-----	-----	Degradation of methanol and amino acids (Brown *et al.* 2003)

Table 1. Comparative functions of glycosomes/peroxisomes in trypanosomatids, mammals and yeast. (---- : No known function associated in the glycosome/peroxisome.)

The biogenesis and origin of peroxisomes, and thus potentially glycosomes, has been a controversial topic for several years. Two models have been suggested for the development of new peroxisomes. The growth and division model implies that early peroxisomes grow into mature peroxisomes and undergo subsequent differentiation. Studies suggest that the growth and division model is prominent in terms of glycosome biogenesis resembling the biogenesis of mitochondria and chloroplast in which division of the mature peroxisomes occurs during cell division (Lazarow PB, 2003). In contrast, the *de novo* synthesis model suggests that pre-peroxisomes originate from an unknown membrane source, possibly the ER, and acquire important PEX proteins ultimately leading to the presence of a mature peroxisome (Purdue and Lazarow, 2001). However, recent studies suggest that a signal from within the peroxisome initiates division which can only occur once the complete set of matrix proteins are present forming a mature peroxisome (Guo *et al.* 2007).

E. Energy production

The glycosomal portion of energy metabolism is redundant in the majority of Trypanosomatids (Tielens and Van Hellemond, 1998). The first seven steps of glycolysis occur within the glycosome leading to the production of 3-phosphoglycerate, which is exported out of the glycosome. Unlike *T. brucei*, which has different energy mechanisms depending on the stage of the parasite, the energy production mechanisms are quite consistent throughout the different stages of the *Leishmania* lifecycle (Tielens and Van Hellemond, 1998). The 3-phosphoglycerate

is converted to pyruvate which is imported into the mitochondria where it will participate in energy production through the Krebs cycle. It has been shown that *Leishmania* promastigotes exhibit an energy metabolism in which a minimal percentage of carbohydrate is completely oxidized to carbon dioxide (Tielens and Van Hellemond, 1998). Instead, large amounts of partially oxidized products such as acetate, pyruvate and succinate are produced as end-products within the Krebs cycle. It is also important to note that energy production with in *Leishmania* is dependent upon the presence of oxygen (Tielens and Van Hellemond, 1998). Due to this constraint *Leishmania* promastigotes have been shown to reduce metabolism to a minimal level in order to tolerate hypoxic conditions (Van Hellemond and Tielens, 1997). This reversible condition is thought to enable the survival of promastigotes in harsh conditions within the sandfly vector as the *Leishmania* promastigotes can survive within a limited nutrient environment.

F. Glycosomal and peroxisomal protein import

Import of proteins across a biological membrane requires specificity which in most cases is regulated by targeting signals in proteins. Different import systems require specific import signals; endoplasmic reticulum targeting encompasses a 16- to 30-residue signal sequence, typically located at the N-terminus of the protein (Loewen et al. 2003). *Leishmania* glycosomes are no different as protein import into the organelle requires one of three specific signal sequences. The signal sequences are referred to as Peroxisomal Targeting Sequence 1 (PTS1), PTS2 and PTS3. The PTS1 import signal incorporates a C-terminal tripeptide that includes the sequences

Ala-Lys-Leu (AKL) or Ser-Lys-Leu (SKL) or variants thereof (Blattner et al. 1992; Lametschwndter et al. 1998). Examples of proteins incorporating this PTS1 topogenic sequence in Leishmania include, inosine monophosphate dehydrogenase (IMPDH), xanthine phosphoribosyl transferase (XPRT) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Opperdoes and Szikora, 2006; Jardim et al. 1999; Wilson et al. 1991; Allen et al. 1989). The PTS2 signal comprises an N-terminal nonapeptide with the sequence R/K-L/I/V-X5-H/E-A/L found within the first 20-30 amino acids (Subramani *et al.* 2000). A prominent PTS2 protein is that of hexokinase (Umansakar *et al.* 2005), which is involved in glycolysis within the glycosome. The PTS3 signal sequence, also referred to as I-PTS, is an internal targeting sequence although little else is known about the mechanism of protein import via PTS3 signals (Small *et al.* 1988; Kragler *et al.* 1993). These signal sequences are conserved in several *Leishmania* spp. (Opperdoes and Szikora, 2006; Shih *et al.* 1998) as well as in the Kinetoplastid *T.* brucei (Blattner *et al.* 1992). Proteins that incorporate either PTS1 or PTS2 import signals are bound by the cytosolic receptor proteins peroxin 5 (PEX5) or PEX7, respectively. Upon binding, the cytosolic receptor complex interfaces with the peroxisomal/glycosomal membrane. PEX14 is a membrane bound protein that forms a docking complex that interacts with a cargo protein loaded PEX5 and PEX7 receptor. Interaction of PEX14 with the cargo loaded receptor results in import of PTS tagged proteins into the glycosome/peroxisome via an unknown mechanism. A transient pore model has been suggested in which PEX5-PTS1 loaded cargo oligomerizes to form a pore which then inserts into the peroxisome membrane allowing the release of the PTS-1

protein into the glycosome (Erdmann and Schliebs, 2005). Following the import of the PTS-1 protein, ubiquitination of PEX5 occurs and it is released from the membrane removing the transient pore. A similar extended shuttle model has been suggested for the other cytosolic receptor PEX7 in yeast (Nair *et al.* 2004). With respect to Leishmania, studies with LdPEX5 suggest that translocation does not occur during protein import into the glycosome, thus a cycling mechanism is absent (Madrid *et al.* 2004).

G. Mammals and yeast

The peroxisomes of yeast and mammals have been studied extensively and approximately 35 PEX proteins are proposed to be involved in the formation of a functional peroxisome (Schrader and Fahimi, 2006). Peroxisomal enzymes are required for β-oxidation of long fatty acid chains, synthesis of cholesterol, ether, phospholipids, and bile acids, as well as the catabolism of phytanic acid and pipecolic acid (Oliviera *et al.* 2002). The relevance of peroxisomes within the mammalian host is well documented due to the occurrence of several human genetic diseases in which one or several peroxisome functions are down regulated. These diseases termed peroxisome biogenesis disorders (PBDs) include, X-linked adrenoleukodystrophy, Zellweger's syndrome, infantile refsum disease and rhizomelic chondrodysplasia punctata type I (Shimozawa, 2007; Brown and Baker, 2003). Recently, a new group of mutations have been observed in the *Homo sapien* PEX14 (HsPEX14) gene leading to the formation of ghost peroxisomes in patients in Japan (Shimozawa, 2006). In yeast, peroxisomes are the sole site of fatty acid β-

oxidation of long chain fatty-acids (> C18). Other pathways in yeast, which involve the peroxisome, include the biosynthesis of lysine and degradation of methanol and amino acids (Brown and Baker, 2003). Thus a wide range of organisms contain peroxisomes/glycosomes in some capacity, their functions are not identical however they do contain similarity with respect to metabolic pathways. The evolutionary tree of the Eukaryotes (Fig. 2) demonstrates that the order Kinetoplastida diverged from the higher Eukaryotes. The divergence that occurred helps to explain the differences and similarities that are observed between the Kinetoplastida and the higher Eukaryotes with respect to the glycosomes/peroxisomes.

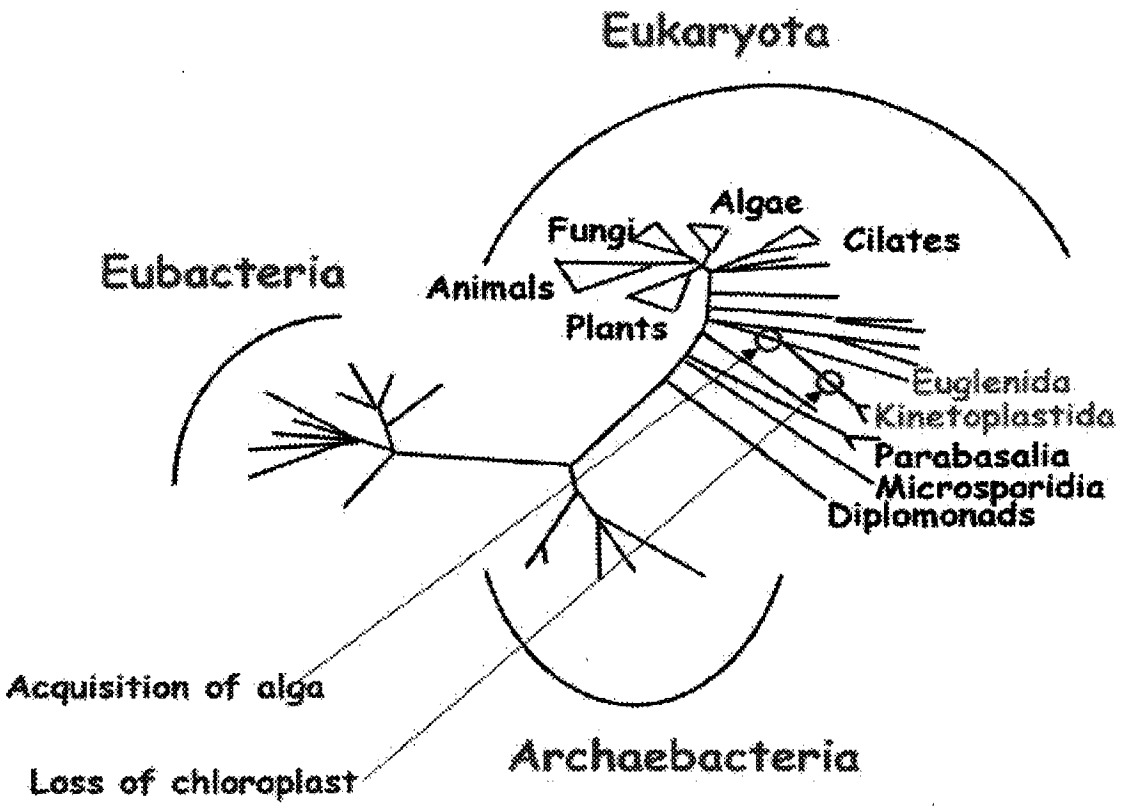

Figure 2. Evolutionary tree. Demonstrating divergence of the order Kinetoplastida from the other Eukaryotic organisms. (Hannaert et al. 2003)

A similar observation can be made with respect to the peroxin proteins involved in peroxisome and glycosome function; there is a lack of conservation across all species. HsPEX14 is the mammalian homologue of *Leishmania* PEX14. Sequence conservation between PEX14 is observed only for a short span at the N-terminus of the protein (Fig. 3, underlined) (Madrid and Jardim, 2005). HsPEX14 is suggested to be an intrinsic peroxisomal membrane protein that is involved in the docking of PEX5. Through protease analysis it was determined that the first 130 amino acids of HsPEX14 are highly protected from degradation, thus possibly implicating those N-terminal residues in peroxisomal binding (Oliviera *et al.* 2002). Further analysis of HsPEX14 has shown that amino acid's 147-278 representing a coiled-coiled motif, may be responsible for HsPEX14 homopolymerisation. Other interactions of PEX14 in mammals include the binding of PEX13 and Pex19. Lower eukaryotes also demonstrate binding of PEX14 to PEX3, PEX7, PEX8 and PEX17, leading to the possibility for many more interactions being observed for LdPEX14. With respect to yeast, ScPEX14 has been shown to form a binding complex with ScPEX13 and ScPEX17. Studies to map the binding region of PEX14 in yeast have revealed that ScPEX14 contains two distinct binding domains for ScPEX5 and ScPEX7 respectively (Niederhoff *et al.* 2005)

Figure 3. Pairwise sequence alignment of the N-terminus of *Leishmania donovani, Trypanosoma brucei, Homo sapiens* and *Saccharomyces cerevisiae* PEX14 using CLUSTALX computer program. An asterisk indicates conserved amino acid sequences while similar amino acid replacements are indicated by dots below the sequence alignment. Underlined segment represents conserved element observed in all PEX14 proteins across species. Predicted transmembrane segment of LdPEX14 (Bold) and the leucine-zipper motif (Bold, double underline)

H. *Trypanosomes*

Trypanosome and *Leishmania* parasites are found in the order Kinetoplastida based on the presence of a kinetoplast within the mitochondria, a specialized DNA-containing organelle. With respect to glycosome function and import, similarities exist between *Leishmania* and *Trypanosoma.* The glycosome, and glycolytic pathway, in *Trypanosomes* is considered an excellent target for drug design as the *T. brucei* bloodstream form generates ATP solely based on production from glycolysis. With respect to the generation of ATP within *Leishmania* the glycolysis pathway is not believed to be the sole source of ATP (Moyersoen *et al.* 2004). The glycosomal import pathway of *T. brucei* has been studied extensively and much has been elucidated, of importance is the fact that ribonucleic acid interference (RNAi) depletion of TbPEX14 mRNA results in mislocalization of glycosomal proteins and death of the parasite (Moyersoen *et al.* 2004; Moyersoen *et al.* 2003; Furuya *et al.* 2002). Since the technique of RNAi does not function in *Leishmania*, together with the lack of regulatable promoter elements, makes the generation of *Leishmania* mutants lacking proteins involved in glycosome biogenesis an arduous task.

Through mutational studies the interaction domains of TbPEX14 and TbPEX5 have been determined. TbPEX14 requires an N-terminal region comprising the first 83 amino acids in order to interact with TbPEX5. Hydrophobic residues located at amino acids F35 and F52 were also shown to be critical for this interaction (Choe *et al.* 2003). With respect to the motifs required within TbPEX5 it was observed that the first and third WXXXY/F motifs were important characteristics for the efficient

binding to TbPEX14 (Choe *et al.* 2003). Other peroxins involved in either glycosome import or biogenesis have been identified recently. TbPEX19, showing 20% homology to the human PEX19, has been shown to be required for insertion of peroxisomal membrane proteins (Banerjee *et al.* 2005). Knockdown mutants of TbPEX19 demonstrated that parasites synthesized fewer glycosomes that were larger (Banerjee *et al.* 2005). TbPEX6, TbPEX10 and TbPEX12 have also been identified. Disruption of these peroxisomal proteins by RNAi demonstrated reduced growth of the parasite. TbPEX6 was expressed and suggested to contain an ATPase associated with various cellular activities, whereas TbPEX10 and TbPEX12 were observed to be integral membrane proteins with both their N- and C-terminals exposed to the cytosolic side (Krazy and Michels, 2006).

I. LdPEX5

LdPEX5 is similar in structure to the other PEX5 homologues, it comprises a bidomain protein in which the C-terminus contains seven tetratricopeptide repeats (TPRs) that form a binding pocket for the PTS-1 signal (Jardim *et al.* 2000). The N-terminal region of LdPEX5 contains sequences that form important interactions for the import of PTS-1 proteins (Madrid *et al.* 2004). Several studies have demonstrated that the N-terminal region contains domains responsible for the interaction with the glycosomal protein PEX14 (Jardim *et al.* 2002).

The employment of a number of biophysical techniques have suggested that in the absence of PTS1-labelled proteins, LdPEX5 oligomerizes forming a tetrameric

structure. Further analysis on the confirmation of LdPEX5 in the presence of PTS1 proteins showed that the tetrameric structure shifts to a dimeric structure upon interaction (Madrid *et al.* 2004). The dimeric LdPEX5-PTS1 complex then interacts with LdPEX14 on the glycosomal membrane.

J. LdPEX14

LdPEX14 is a 464 amino acid peripheral membrane associated protein that is anchored to the cytosolic face of the glycosomal membrane. The amino acid sequence of mammalian, yeast and plant are predicted to contain a single putative transmembrane domain. The *L. donovani* PEX14 is proposed to have a hydrophobic domain at the N-terminus from amino acids 148-176 (Fig. 3, bold transmembrane domain). Analysis of LdPEX14, using Triton X-114 extraction and protease K treatment suggests that the entire LdPEX14 molecule is exposed to the cytosol (Jardim *et al.* 2002). The LdPEX14 primary sequence contains a predicted leucine zipper motif, amino acids 274-316, which has been suggested to be involved in homopolymerization of LdPEX14 on the glycosomal membrane (Fig. 3, double underline) (Itoh and Fujiki, 2006; Snyder *et al.* 1999). Therefore, LdPEX14 conceivably oligomerizes on the glycosomal membrane allowing for the formation of a docking complex that interacts with the LdPEX5-PTS1 protein complex. It is important to note that the PEX14 protein family is poorly conserved, showing approximately 10% sequence identity across phylogeny (Madrid and Jardim, 2005). The only significant sequence retained among the PEX14 proteins is an N-terminal motif comprising the sequence A-X2-F-L-X7-S-P-X6-F-L-K-G-K-G-L/V (Fig. 3,

underline, * indicates conserved amino acids). Studies using the *Arabidopsis* PEX14 (Nito *et al.* 2002) and *T. brucei* PEX14 (Choe *et al.* 2003) demonstrated that this conserved sequence is critical for binding to the WXXXY/F pentapeptide domains of PEX5. Through mutational analysis it has been established that the N-terminal region, from amino acids 23-63 of LdPEX14 encompasses a critical motif responsible for the interaction with LdPEX5 (Madrid and Jardim, 2005). Similar experiments involving human PEX14-PEX5 interactions have suggested that a region in the first 78 amino acids of HsPEX14 is important for this interaction (Schliebs *et al.* 1999). With respect to the interaction between PEX14 and PEX5 on the glycosomal surface it has been suggested that LdPEX14 interacts with LdPEX5 with a Kd of 2.75 μM. However, when LdPEX5 complexes with a PTS-1 containing protein the affinity for LdPEX14 increases to 398 nM (Jardim *et al.* 2002). However, little is known about the motifs by which LdPEX14 is attached to the glycosomal membrane. Studies involving human PEX14 have shown that N - terminal residues 21-140, including a hydrophobic segment at 110-138, function as a topogenic sequence (Itoh and Fujiki, 2006).

III. THESIS OBJECTIVES

This thesis focuses on understanding the interactions of LdPEX14 with the glycosomal membrane. This would allow further characterization of the glycosomal import pathway in *Leishmania.* The specific objectives for this project are:

1. Characterize the interaction of LdPEX14 N-terminal mutants with the glycosomal membrane.

2. Examine the growth of different LdPEX14 mutants and the effects on cell viability

IV. MATERIALS AND METHODS

A. *Leishmania* PEX14 mutant constructs

Epitope-tagged LdPEX14 – The LdPEX14 open reading frame (ORF) was amplified by PCR using the sense primer (LdPEX14-FLAG) 5'-CATGCCATGGACTACAAG GACGACGACGACA AGATGGCGGCAG AAGTCC-3' containing an *Nco*I site (underlined), and the antisense primer (LdPEX14-HA) 5'CGGGATCCTAAGCGTA GTCTGGGACGTCGTATGGGTAGCCAATCGACATCGG-3' containing a *BamH* I site (underlined) to generate an N-terminal FLAG and C-terminal hemagglutinin (HA) epitope tagged form of LdPEX14 (FLAG-LdPEX14-HA). Amplification was performed using *Pwo* polymerase (Roche Molecular Biochemicals, Indianapolis, IN), with 20 cycles of denaturation at 94 ^{0}C for 45 s, annealing at 52 ^{0}C for 45 s and extension at 72 ^{0}C for 3 min. The FLAG-LdPEX14-HA PCR fragment was digested with *Nco* I/*BamH* I and the ends were filled-in with T4 DNA polymerase and ligated into the *Leishmania* expression vector pALT-NEO (Tobin et al. 1991) The pALT-NEO vector was previously digested with *Xba* I and the ends filled-in with T4 DNA polymerase to generate the construct pALT-NEO-FLAG-*LdPEX*14-HA. The pALT-NEO-His$_6$/S-*ldpex*14Δ 1-63-HA construct was generated by cloning the *Xba* I/*Pst* I fragment from pET30b(+)-His$_6$/S-FLAG-*LdPEX14*-HA into the corresponding sites of the pET30b(+)-His$_6$/S-*ldpex*14Δ 1-63. The pET30b(+)-His$_6$/S-*ldpex*14Δ 1-63-HA construct was digested with *Nde* I/*BamH* I and the 1.5 kb fragment was treated with *Pfx* polymerase to fill-in the ends and then cloned into the pALT-NEO vector prepared as above. The ldpex14Δ1–23-HA mutant was constructed using the sense primer, LdPEX14Δ 1-23, 5'ATGGATTACAAGGATGATGATGATAAGCA

GCCGTCTTCGTCGGAAC3' and the antisense primer 5'CGGGATCCTAAG CGTAGTCTGGGACGTCGTATG GTAGCCAATCGACATCGG 3' on the pALT-NEO-*LdPEX14* template. The PCR products were ligated into PCR 2.1 vector using the restriction endonucleases *EcoRI* and *SacI*. The PCR products were then cloned into pALT-NEO by excising the fragment using *EcoR V* and joining it into pALT-NEO using the restriction endonucleases *SSTI* and *BSTXI*.

The pALT-NEO-FLAG-*ldpex*14 1-119 construct was generated by introducing a stop codon downstream of residue 119 by site-directed mutagenesis using the primer pairs 5'-CGGCAAAACGCGTAAGTGACC ATGACG- 3' with 5'-CGTCATGGT CACTTACGCGTTTTGCCG- 3', and 5'-CGTCA TGGTCACTTACGCGTTTT GCCG- 3' with 5'-CGCTGTCGTCTGCTAGGCCTCGC CGAC-3', respectively.

The pALT-NEO-FLAG-*ldpex*14 1-74-GFP construct was generated by ligating the *GFP* ORF into pALT-NEO-FLAG-*ldpex*14-HA vector digested with *EcoN* I and polished with T4 DNA polymerase. The pALT-NEO-*GFP* and pALT-NEO-*GFP-AKL* constructs were generated by PCR using the above GFP-sense primer and the antisense primers (GFP-antisense) 5'-GTGTCTAGATTAATCCATGCCATG-3' and 5'-GCTGCTCTAGATTATAACTTG GCATC-3' containing an *Xba* I site (underlined). The PCR products were cloned into the *Bam*HI/*Xba*I sites in the pALT-NEO vector using a blunt-sticky end strategy. All constructs were verified by restricted digestion and automated DNA sequence analysis.

B. Cell culture

L. donovani cells were grown at 26 ^{0}C in Dulbecco's Modified Eagle- *Leishmania* (DME-L) media supplemented with 5% FBS, 5 mg/ml hemin (prepared in 0.5M NaOH), 100 μM Xanthine and 1 ml/L triethanolamine.

C. Transfection of *Leishmania* parasites

Wildtype *L. donovani* promastigotes (2 x 10^8 cells) were harvested by centrifugation at 3, 000 rpm, 10 min and washed once in 10 ml of electroporation buffer (21 mM Hepes, 137 mM NaCl, 5 mM KCl , 0.7 mM Na_2HPO_4, 6 mM glucose). The cell pellet was resuspended in 2.0 ml of electroporation buffer and 0.4 ml of cell suspension was transferred into a Bio-Rad electroporation cuvette (0.4 cm). 10 μg of sterile plasmid DNA was added to the cell suspension and the cuvettes were incubated on ice for 10 min prior to electroporation using the parameters 0.45 kV, and 500 μF on a Bio-RAD Gene Pulser II. Following electroporation, the cell suspension was transferred into a 25 ml culture flask and the cuvette was washed with 1 ml complete DME-L media. 5.0 ml DME-L media was added to the flask and the cultures were incubated at 26 ^{0}C for 48 hrs and cell viability was analyzed by microscopy. Parasites that were successfully transfected were selected by adding 20 μg/ml G418 (Neomycin) (Fisher Scientific).

D. Immunofluoresence confocal microscopy

Logarithmic phase *L. donovani* promastigotes and transfected promastigotes were harvested, washed with PBS and resuspended at 1 x 10^7 cells/ml in PBS. Cells were attached to poly-L-lysine coated coverslips (0.1% lysine solution) for 15 min. Cells were fixed with 4% paraformaldehyde for 10 min at 20 ^{0}C and washed twice with PBS. Cells were permeabilized and blocked with PBS/0.25% Triton X-100, 3% Bovine serum albumin (BSA) for 30 min and then incubated with primary antibodies, rabbit anti-LdPEX14 (1:1, 000), guinea pig anti-LdIMPDH(1:500), or rat anti-HA-fluorescein (1:100) (Hoffman-La Roche) for 1 h at 20 ^{0}C. Coverslips were washed 4X 2.0 ml PBS then incubated with a goat anti-rabbit IgG-Cy3 (1:1, 500) (Amersham Biosciences, Cedarlane) or goat anti-guinea-pig IgG-FITC (1:500) (Jackson Immuno Research) in PBS/3% BSA for 1 h. Coverslips were washed 5X 2.0 ml PBS to remove unbound secondary antibody and mounted on slides using n-propyl gallate as the anti-quenching agent. Slides were analyzed using a Bio-Rad Radiance 2000 confocal microscope.

E. Subcellular fractionation

Silicon Carbide cell disruption

L. donovani promastigotes (2 x 10^{10} cells/ml) were harvested by centrifugation at 5, 000 rpm for 10 min. The cells were washed twice with 2.5 ml cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris pH 8.0, 1 mM DTT, protease inhibitor cocktail(Hoffman-Roche)) and centrifuged at 5, 000 rpm for 15 mins. The parasites were manually homogenized by mixing with an equal volume of silicon

carbide in homogenization buffer in a Dounce homogenizer. The percentage of cell lysis was monitored microscopically until approximately 90% of the cells had been lysed. The homogenate was centrifuged at 1, 500 rpm for 15 mins at 4 ^{0}C to remove unbroken cells, silicon carbide and cell debris. The remaining supernatant was centrifuged at 3, 000 rpm for 10 mins at 4 ^{0}C, producing a heavy mitochondrial pellet which was discarded. The supernatant was centrifuged at 40, 000 *x g* for 40 mins to produce a light mitochondrial pellet and resuspended in 4.0 ml homogenization buffer and overlaid on a 25-60% discontinuous sucrose gradient and centrifuged on a Beckman type SW41 swinging bucket rotor at 29, 500 rpm for 17 hr at 4 ^{0}C. The gradient was fractionated into 500 µL fractions collected from the top. The fractions were run on an SDS-PAGE gel for Western blot analysis. In sucrose gradients, glycosomes tend to sediment near the lower end of the gradient at approximately 55% sucrose (Jardim *et al.* 2000). An antibody raised against XPRT (Rabbit 1:1, 000; secondary 1:10, 000) was used as an indicator of glycosomal localization. Antibodies raised against GFP (Rabbit 1:300; secondary 1:10, 000) (Cedarlane) and FLAG (Rabbit 1:5, 000, secondary 1:10, 000) (SIGMA) were used to determine the localization of FLAG-ldpex 1-74-GFP. Antibodies raised against Hemagglutinin (HA) (Rat 1:200, secondary 1:2, 000) (Hoffman-Roche) were used to examine the localization of ldpex14Δ 1-63-HA. Antibodies raised against the Human ubiquitin 20S proteasome (Rabbit 1:1, 000, secondary 1:10, 000) (Chemicon) were used to visualize the *Leishmania* proteasome.

Hypotonic Lysis

Alternatively, cell lysis was done by harvesting mid-log phase L. donovani promastigotes (3 x 10^9 cells) and re-suspending cells in cold hypotonic buffer (5 mM HEPES pH 7.4, 2 mM EGTA, 2 mM DTT, protease inhibitor cocktail) at a density of 2 x 10^8 cells/ml and lysed with 20 passes through a 27 gauge needle. Lysates were made isotonic by adding 0.25 volumes of a 50 mM HEPES pH 7.4, 0.25 M sucrose, 1 mM ATP, 1 mM EGTA, protease inhibitor cocktail solution and centrifuged at 3, 000 x g for 15 min at 4 ^{0}C. Postnuclear lysates (5.0 ml) were layered onto an 8.0 ml 20%-60% linear sucrose gradient with a 70% cushion and samples subjected to centrifugation at 218, 000 x g for 6 h at 4 ^{0}C in a Beckman SW41 rotor. Gradients were fractionated from the top (500 µl/fraction) and 150 µl of each fraction was precipitated with methanol-chloroform prior to analysis by Western blot. For 1% igepal extractions, cells were treated on ice for 15 mins following the 3, 000 x g spin with 1% igepal prior to loading onto the sucrose gradient.

F. Glycosomal Preparation (Nycodenz)

Logarithmic phase *L. donovani* promastigotes (5 x 10^8 cells) were harvested by centrifugation at 3, 000 rpm for 10 mins. Cells were washed twice with 10 ml homogenization buffer (HB) (0.25 M sucrose, 1 mM EDTA, 10 mM Tris pH 8.0, 1 mM DTT and protease inhibitors). Cells were resuspended in 1.5 ml of homogenization buffer and transferred to a 2.0 ml tissue grinder. An equal volume of silicon carbide was added (~2.5 grams) and cells were homogenized to 90% lysis.

The homogenate was spun at 5, 000 rpm for 10 mins at 4 ^{0}C to remove nuclei and unbroken cells. The supernatant was removed and layered onto 650 µl of 30% Nycodenz in a thick wall ultracentrifuge Eppendorf tube (Beckman). Tubes were centrifuged at 49, 000 rpm for 1 hr at 4 ^{0}C. Following centrifugation, 300 µl of the supernatant was transferred to a new Eppendorf tube and the proteins precipitated by trichloroacetic acid (TCA). Residual supernatant was removed by overlaying 300 µl of HB over the Nycodenz layer. The HB buffer was carefully removed and the process was repeated once more. The remaining volume of Nycodenz was removed from the bottom of the Eppendorf and the glycosomal pellet was re-suspended in 30 µl of SDS-PAGE sample buffer. Samples were resolved on SDS-PAGE and probed with anti-LdPEX14 (as described above).

G. Digitonin extraction

L. donovani promastigotes (2.5 x 10^9 cells) were harvested, washed with PBS, and resuspended in 2.5 ml of fresh STES buffer (0.25 M sucrose, 25 mM Tris pH 7.4, 0.15 M NaCl, 1 mM EDTA). An aliquot of the cell suspension (100 µl) was mixed with 250 µl digitonin in STES buffer (0 mg/ml to 0.36 mg/ml). The cell suspension was incubated for 5 mins at room temperature, then placed on ice for 5 min prior to centrifugation at 13, 000 rpm for 2 minutes. The supernatant was removed and transferred to a new Eppendorf tube. A Bradford assay (Bio-Rad) was performed to determine the protein concentrations in the supernatants. The proteins were then precipitated with TCA, and the pellets resuspended in 75 µl of SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE and analyzed by Western blot.

H. Growth Curves

Parasite cultures containing wildtype, FLAG-LdPEX14-HA and FLAG-ldpex14 1-119 transgenic *L. donovani* parasites were prepared by seeding 5 x 10^5 cells/ml in culture flasks containing 50 ml of DME-L culture media with 20 µg/ml G418. Parasites were counted every second day at early time points and subsequently counted daily during logarithmic phase. All counts were performed in triplicate for each time point and the mean plotted as a function of culture age.

I. Protein Expression and Purification of His/S-LdPEX14-HA

E. coli ER2566 cells transformed with pET30(b)-His/S-*LdPEX*14-HA were grown to OD 0.7 at 37 ^{0}C. Protein expression was induced with 0.7 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 20 ^{0}C for 3 hrs. Cells were harvested and the pellet resuspended in 10 ml of buffer 1 (20 mM Tris, pH 8.0, 0.25 M NaCl) and cells were lysed with two passes of a French press. Lysates were clarified by centrifugation at 12, 000 rpm for 30 min and the supernatant was loaded onto a Ni^{2+}-NTA column (Qiagen). The column was washed with 100 ml wash buffer (40 mM Tris pH 8.0, 0.5 M NaCl and 10 mM imidazole). The recombinant protein was eluted using an imidazole step gradient in wash buffer (20 mM, 40 mM, 80 mM and 320 mM imidazole). The bulk of the recombinant protein was recovered in the 80 mM imidazole fractions. Fractions were concentrated using an Amicon Biomax 5K NMWL centrifugal filter (Millipore, Bedford, MA, U.S.A.) and samples were run on SDS-PAGE to ensure purity of the sample.

J. Giemsa Stain

To compare the cellular morphology of transgenic promastigotes with the *L. donovani* wildtype parasites, 1 ml of FLAG-LdPEX14-HA and FLAG-ldpex14 1-119 promastigotes were obtained, washed and resuspended in PBS. 15 µl of the cell suspension was then added to a slide and allowed to air-dry. Cells were fixed by immersing the slides in methanol for 30 s and stained with giemsa for 10 mins at a 1:10 dilution from the stock solution (Fisher scientific). Slides were then washed with dH_2O. Parasite size was measured using a normal light microscope containing a calibrated ruler.

K. Alamar Blue Assay

FLAG-ldpex14 1-119 (20 µg/ml and 100 µg/ml G418) and FLAG-LdPEX14-HA (100 µg/ml G418) transgenic parasites (1 x 10^7 cells/ml) were harvested and re-suspended in 80 µl of DME-L media. Cells were transferred into a 96-well plate and 20 µl of alamar blue (US Biological) was added to each well and incubated at 26 ^{0}C. Reduction of alamar blue was monitored in a fluorimeter (545 nm excitation and 590 nm emission) after 4 and 24 hrs using a Flexi-dock II station (Molecular Devices). Assays were performed in triplicate for each cell line.

V. RESULTS

Glycosomes in *Leishmania* have several important functions required for parasite survival. Understanding the processes by which the organelle functions is imperative to possible drug development against *Leishmania*. The PTS1 import pathway involving LdPEX5 and LdPEX14 shows resemblance to the mechanism of glycosomal import in *Trypanosomes*. LdPEX14 has been shown to be unique when compared to yeast and mammalian homologues. In the latter case, human PEX14 (HsPEX14) is an integral membrane protein with the first 130 amino acids found within the glycosomal membrane (Oliviera *et al.* 2002). Previous studies involving LdPEX14 have shown that it is a peripherally-associated membrane protein located on the cytosolic face of the glycosomal membrane (Jardim *et al.* 2002). The difference in the topology of binding between the mammalian and *Leishmania* PEX14 is not surprising considering that there is a lack of conservation across the PEX14 protein family with the exception of residues 37-62 showing minimal conservation (Fig. 3, underlined).

In order to determine the binding pattern of LdPEX14 to the glycosomal membrane, several LdPEX14 N-terminal mutants were generated (Fig. 4). These included ldpex14Δ 1-23-HA lacking the first 23 amino acids and ldpex14Δ 1-63-HA lacking the first 63 amino acids encompassing the conserved region of the LdPEX14 protein. In order to further evaluate the binding of LdPEX14 to the glycosomal membrane, two more LdPEX14 mutants were examined, FLAG-ldpex14 1-74-GFP containing the first 74 amino acids of PEX14 and FLAG-ldpex14 1-119 protein construct

encompassing the first 119 amino acids. All protein constructs where expressed extrachromosomally in *L. donovani* transfected parasites using the pALT-NEO expression vector grown in 20 µg/ml G418 up to 200 µg/ml G418.

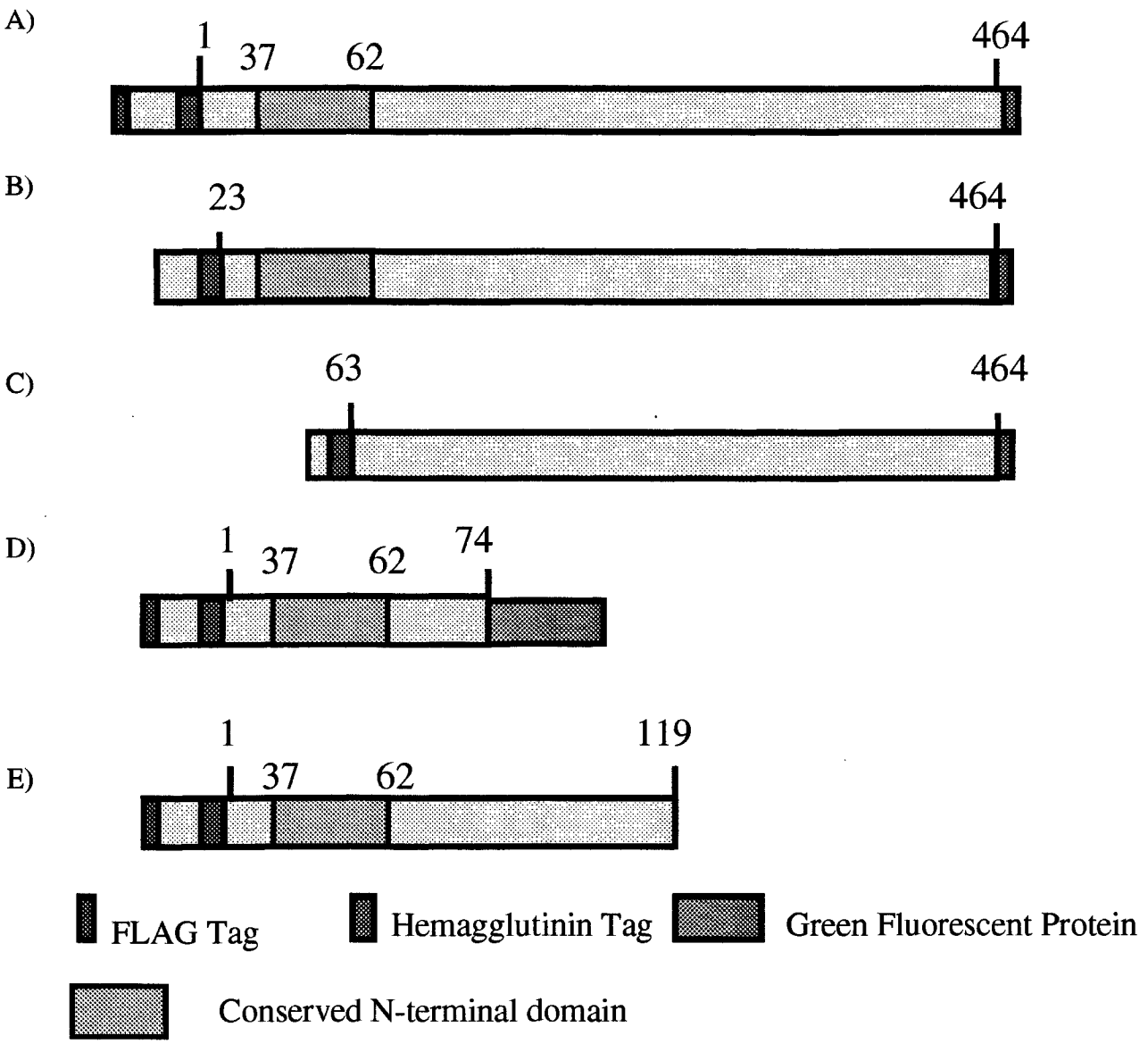

Figure 4. Schematic diagram of LdPEX14 mutant protein constructs transfected into the promastigotes of *Leishmania donovani*. (A) Full Length FLAG-LdPEX14-HA, (B) ldpex14Δ 1-23-HA, (C) ldpex14Δ 1-63HA, (D) FLAG-ldpex14 1-74-GFP and (E) FLAG-ldpex14 1-119.

Localization of tagged LdPEX14

In order to evaluate the localization of the mutant proteins, it was important to establish that the localization of full length recombinant LdPEX14 was unaltered by the addition of N or C-terminal tags. A FLAG Tag, DYKDDDDK, was added to the N-terminus of the full length LdPEX14 and a Hemagglutinin tag (HA) was added to the C-terminus of the full length protein. The localization of FLAG-LdPEX14-HA was monitored via confocal immunofluorescence microscopy (Fig. 5). The recombinant FLAG-LdPEX14-HA was shown to exhibit a punctate staining pattern when probed with anti-HA, indicative of glycosomal localization (Fig. 5, *panel* E). Co-staining with anti-LdPEX14 also demonstrated a punctate pattern (Fig. 5, panel D) similar to that observed with wildtype cells stained with anti-LdPEX14 (Fig. 5, *panel* A). When the images were merged, co-localization of the green and red signals are observed indicating that the FLAG-LdPEX14-HA is faithfully targeted to the glycosomal membrane.

To further confirm these observations, parasites transfected with pALT-NEO FLAG-*LdPEX*14-HA (Fig. 6) were fractionated in a sucrose density gradient. Fractions probed with anti-HA antibodies showed that the signal coincides with the organellar fractions containing glycosomes. This was confirmed by using antibodies to the LdPEX14. These results suggest that the presence of the N-terminal FLAG or C-terminal HA tags did not alter the localization of the epitope- tagged LdPEX14.

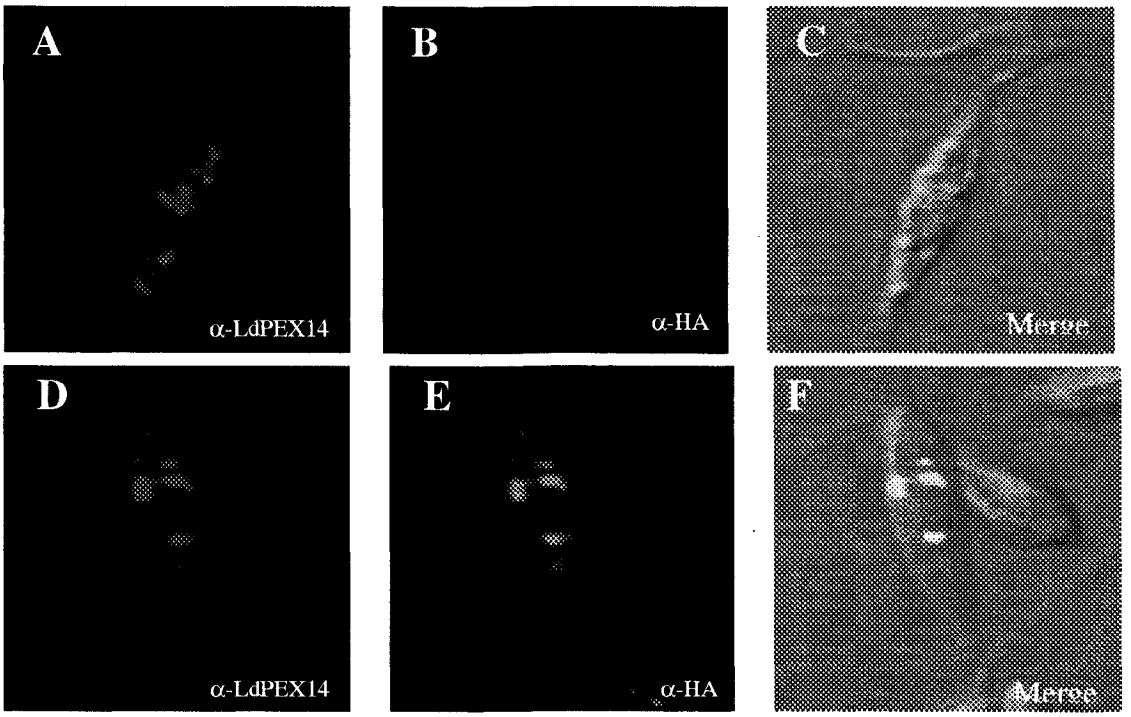

Figure 5. *In vivo* immunolocalization of epitope tagged-LdPEX14 in *L. donovani*. Wildtype L. donovani (A, B, & C) and promastigotes expressing FLAG-LdPEX14-HA (D, E, & F). Slides were immunostained with rabbit anti-LdPEX14 (A & D) and fluorescein-conjugated rat anti-HA antibodies (B & E). Slides were stained with Cy3-conjugated goat anti-rabbit IgG secondary antibodies. Panels C & F represent images resulting from the merging of anti-LdPEX14 and anti-HA respectively.

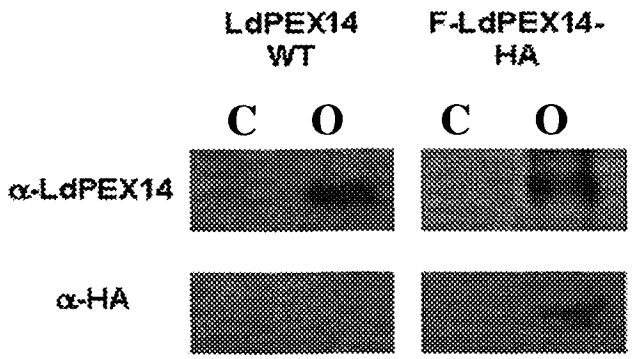

Figure 6. Subcellular localization of FLAG-LdPEX14-HA. Parasites transfected with pALT-NEO FLAG-*LdPEX*14-HA lysed in a dounce homogenizer and the post-nuclear supernatant layered onto a 30% Nycodenz cushion. The upper layer containing the cytosolic fraction (C) and the organellar pellet (O) containing the glycosomes were analyzed by Western blot using anti-LdPEX14 and anti-HA antibodies.

Localization of ldpex14Δ 1-63-HA

Functional studies performed on HsPEX14 revealed that the N-terminal region of the protein was important for targeting to the peroxisomal membrane (Itoh and Fujiki, 2006). In order to determine if the conserved region in LdPEX14 from amino acids 37-63 was required for targeting of LdPEX14 to the glycosome, a mutant protein lacking the first 63 amino acids was generated, ldpex14Δ 1-63-HA (Fig. 4). The open reading frame encoding this recombinant protein was transfected into *L. donovani* promastigotes using the pALT-NEO episomal element to evaluate subcellular localization. Confocal immunofluorescence microscopy experiments showed that pALT-NEO-*ldpex*14Δ 1-63-HA transgenic promastigotes probed with anti-HA antibodies showed staining throughout the cytosolic compartment (Fig.7 *panels* A, B, C) while probing with anti-LdPEX14 showed a punctate staining pattern denoting glycosomal localization. It is important to note that the ldpex14Δ 1-63-HA protein is not recognized by the anti-LdPEX14 antibodies as these recognize epitopes at the N-terminal portion of the protein between amino acid residues 23 and 63. When the anti-LdPEX14 and anti-HA images are merged, no co-localization is observed indicating that the ldpex14Δ 1-63-HA protein is located exclusively in the cytosol. Control experiments using transgenic parasites expressing FLAG-LdPEX14-HA demonstrated co-localization of anti-LdPEX14 and anti-HA signals indicating faithful glycosomal localization (Fig. 7 *panels* D, E, F).

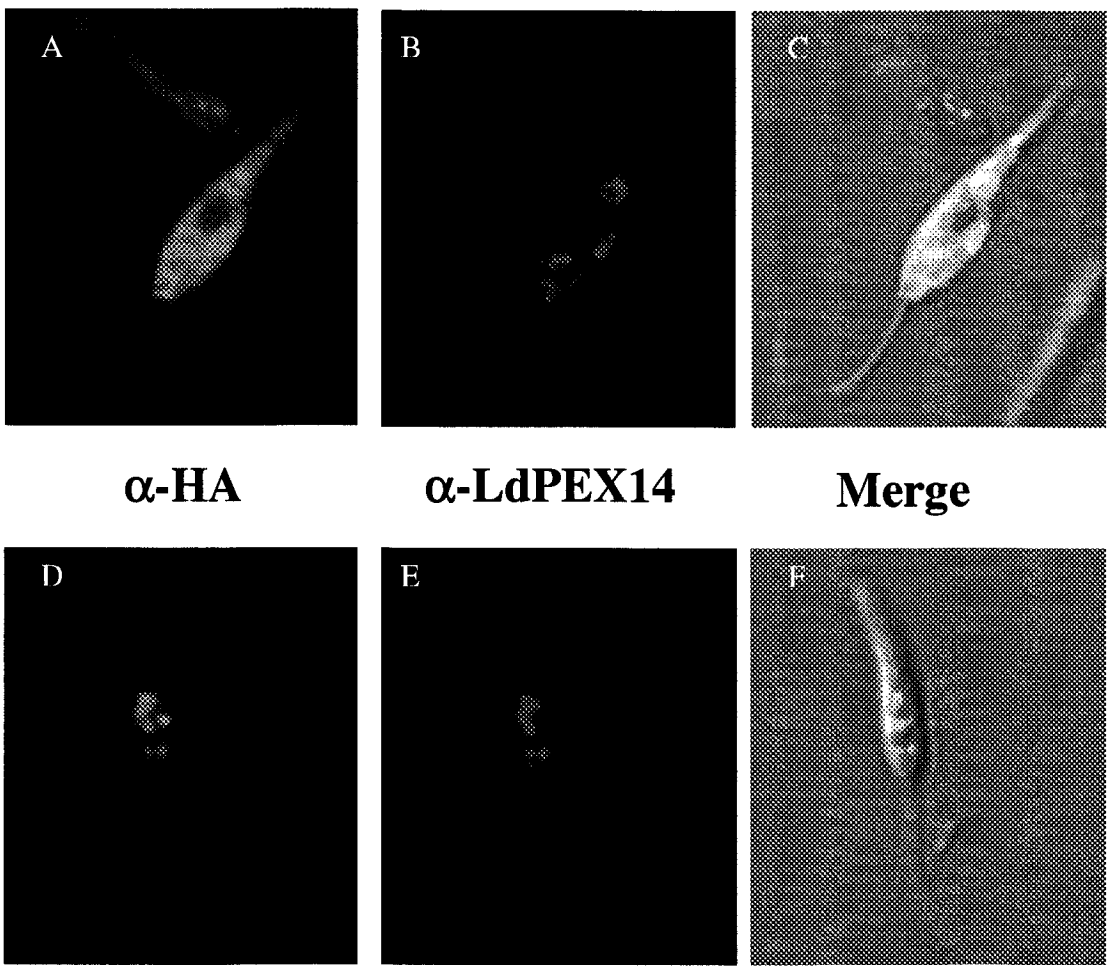

Figure 7. Confocal immunofluorescence microscopy on ldpex14Δ 1-63-HA transgenic promastigotes. (A) Cytosolic localization of ldpex14Δ 1-63-HA in promastigotes stained with anti-HA and FITC antibodies . (B) Punctate glycosomal localization of LdPEX14 stained with anti-LdPEX14 and Cy3 antibodies. The merged image (C) demonstrates that the two signals do not co-localize. FLAG-LdPEX14-HA transgenic promastigotes were used as a control. (D and E) Punctate glycosomal localization in promastigotes stained with anti-HA-FITC and anti-LdPEX14 antibodies. (F) The merged image showed co-localization of the two signals indicating that full length FLAG-LdPEX14-HA is localized to the glycosome.

To confirm that the localization of ldpex14Δ 1-63-HA is cytosolic, subcellular fractionation experiments were performed. Results show that the HA tag associated only with the cytosolic fractions (Fig. 8 *lanes* B and C). The glycosomal marker

protein XPRT (Zarella-Boitz *et al.* 2004) revealed that the glycosomes were present in the lower regions of the sucrose gradient (Fig. 8). The confocal immunofluorescence microscopic analysis and subcellular fractionation experiments suggest that the loss of amino acids 1 to 63 has eliminated the localization of LdPEX14 to the glycosomal membrane.

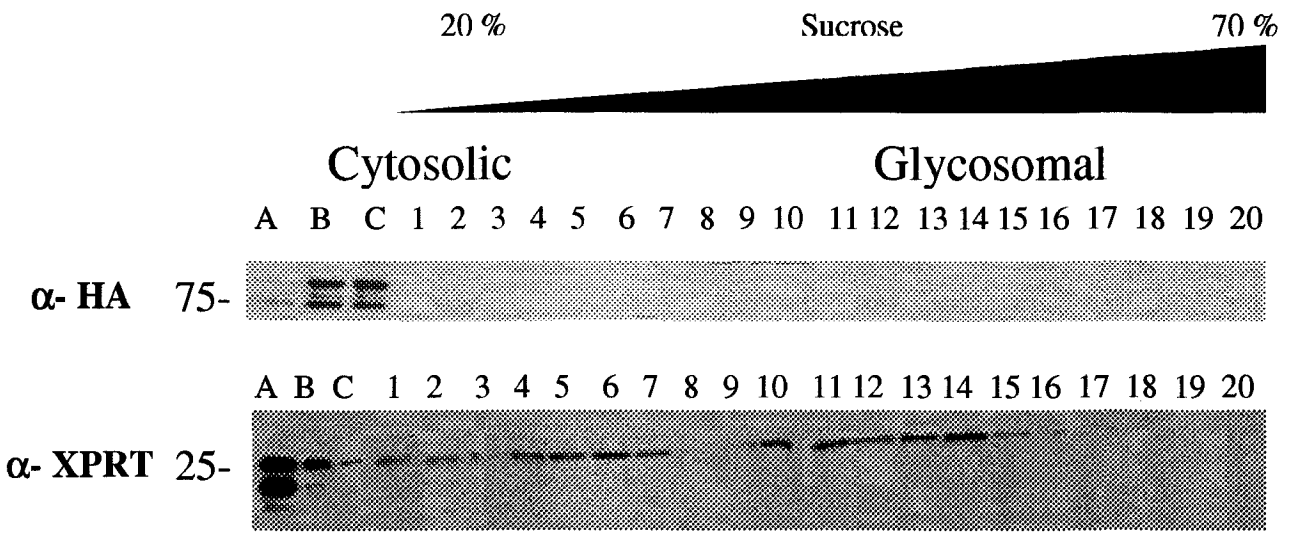

Figure 8. Subcellular fractionation of ldpex14Δ 1-63-HA transgenic promastigotes. (A: 3, 000 rpm Pellet, B: 3, 000 rpm supernatant, C: 40, 000 x g supernatant, 1-21 Sucrose fractions, P: Pellet). The anti-HA signal presents a band above 75 kDa representing the ldpex14Δ 1-63-HA protein found in the cytosolic fractions. The anti-XPRT signal is used as glycosomal marker (25 kDa).

Localization of ldpex14Δ 1-23-HA

The second construct examined was the ldpex14Δ 1-23-HA mutant protein. Expression of ldpex14Δ 1-23-HA was confirmed by probing transfected parasites with anti-LdPEX14 and anti-HA antibodies. Western blot analysis with anti-LdPEX14 on the transgenic parasites showed the presence of a doublet (Fig. 9). The

lower band also cross-reacted with anti-HA confirming that this band was the ldpex14Δ 1-23-HA protein (Fig. 9).

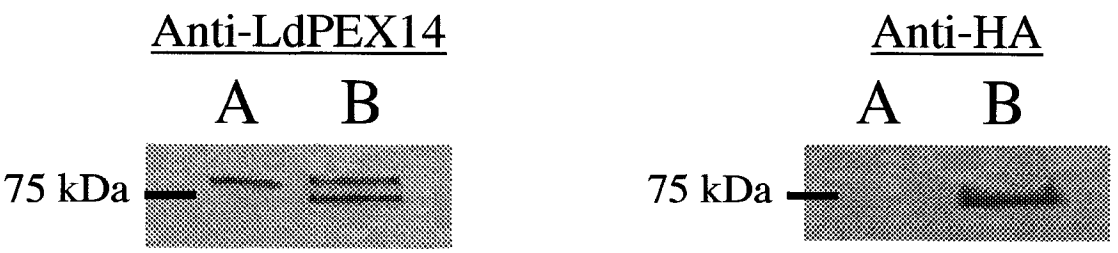

Figure 9. ldpex14Δ 1-23-HA expression. Western blot analysis of (A) wildtype and (B) ldpex14Δ 1-23-HA transgenic parasite lysates using anti-LdPEX14 antibodies showed two bands, one for the native LdPEX14 and the second for ldpex14Δ 1-23-HA. Only a single band is observed when in ldpex14Δ 1-23-HA lysates using anti-HA antibodies.

Confocal immunofluorescence microscopic analysis was performed to determine the subcellular localization of ldpex14Δ 1-23-HA (Fig. 10). ldpex14Δ 1-23-HA appeared to have a dual subcellular distribution when stained with anti-HA and FITC antibodies (Fig. 10, *panel* A). Some of the ldpex14Δ 1-23-HA protein localized to the cytosol, while the remainder of the protein appeared to exhibit a punctuate staining pattern denoting glycosomal localization. This result indicates that the first 23 amino acids are necessary for efficient binding of LdPEX14 to the glycosomal membrane in *Leishmania*. When ldpex14 Δ1-23-HA transgenic parasites were immunostained for IMPDH a dual localization was also observed as shown by the diffuse staining in the cytosol and punctuate glycosomal staining (Fig. 10, *panel* B) whereas wildtype parasites stained with anti-IMPDH and Cy3 showed no diffuse cytosolic staining (Fig. 10, *panel* E).

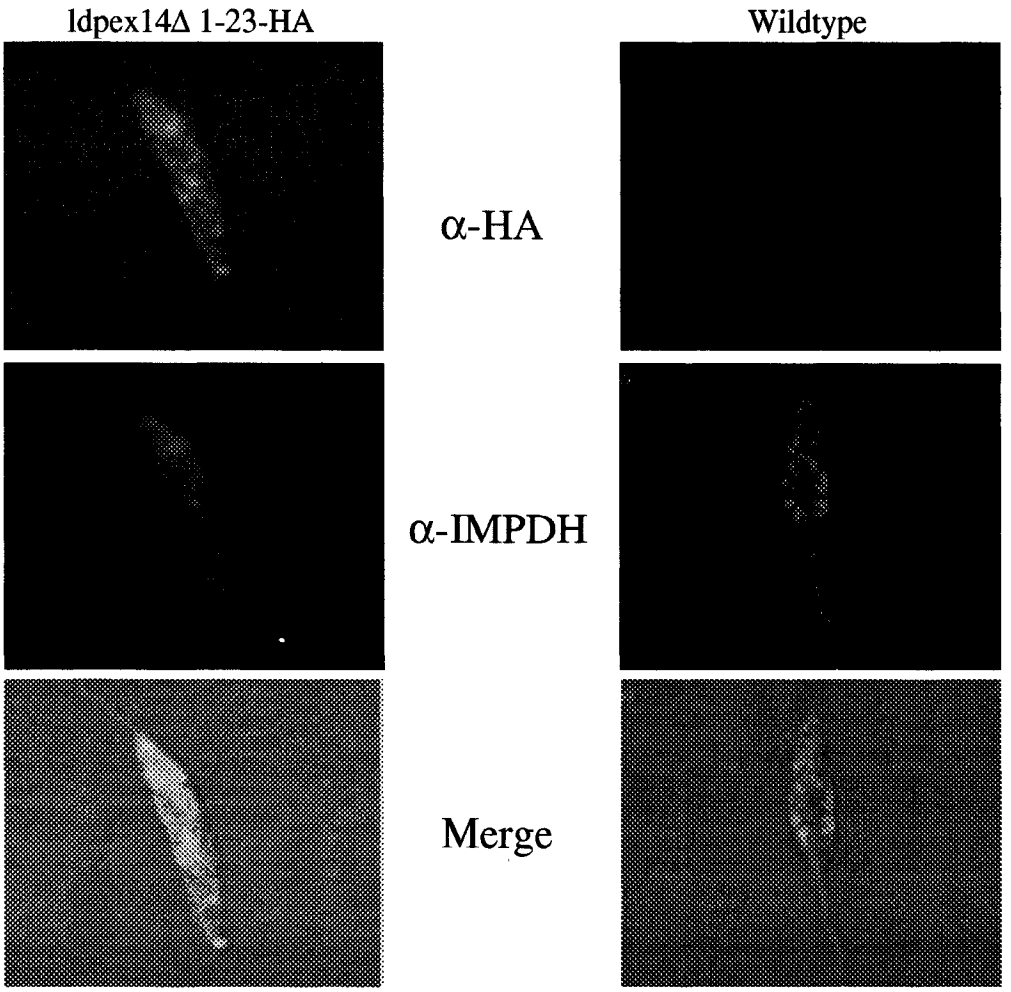

Figure 10. Confocal immunofluorescence microscopy on ldpex14Δ 1-23-HA transgenic promastigotes. (A and B) Dual localization of both ldpex14Δ 1-23-HA and the glycosomal marker IMPDH in transgenic promastigotes stained with anti-HA and FITC antibodies and anti-IMPDH and Cy3 antibodies, respectively. The merged image (C) demonstrates that the two signals co-localize to some extent indicating that the ldpex14Δ 1-23-HA protein is found in both the cytosolic and glycosomal compartments. (D) Wildtype promastigotes stained with anti-HA and FITC antibodies (E) Glycosomal staining in WT promastigotes stained with anti-IMPDH and Cy3 antibodies. (F) Merged image.

To confirm that ldpex14Δ 1-23-HA was dually localized within the parasite, subcellular fractionation experiments were performed using a linear sucrose density gradient. Western blot analysis of the fractions using anti-HA antibodies revealed that ldpex14 Δ1-23-HA was present in both the cytosolic and the glycosomal fractions (Fig. 11 *lanes 7-23*). Similarly, IMPDH also associated with glycosomal and cytosolic fractions confirming the mis-targeting of IMPDH. The cytosolic protein adenine phosphoribosyltransferase (APRT) (Allen *et al.* 1989) was observed to localize only to the cytosolic region of the gradient indicating that the glycosomes were effectively separated from the cytosolic portion (Fig. 11 *lanes 1-7*).

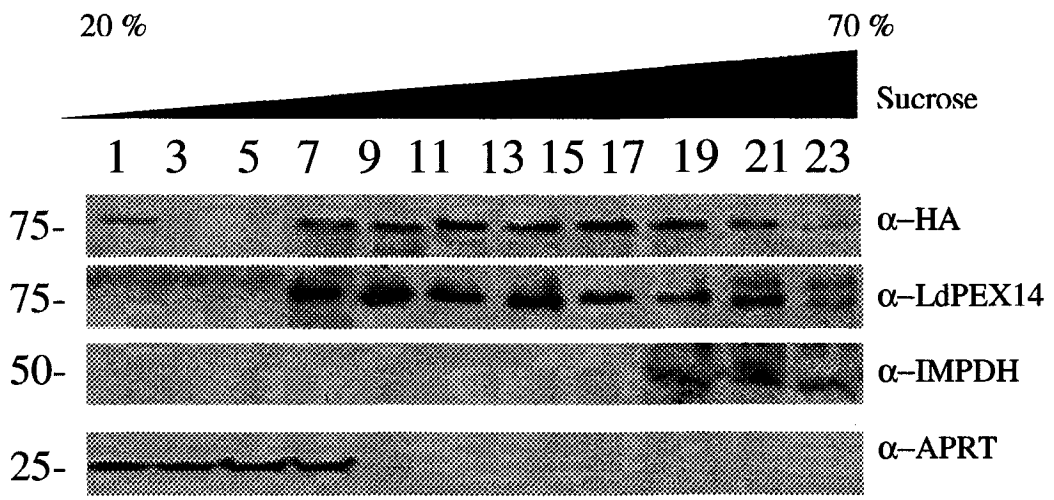

Figure 11. Sucrose density gradient fractionation experiments on ldpex14Δ 1-23-HA. The anti-HA signal is observed throughout the gradient from fractions 7-23. The anti-PEX14 signal recognizes both the native LdPEX14 fractions (*lanes* 19-23) and the recombinant ldpex14Δ 1-23-HA in fractions 7-23. The IMPDH signal is localized chiefly to the fractions containing glycosomes (*lanes* 19-23). The cytosolic marker APRT is localized in the cytosolic fractions (*lanes* 1-7).

Similar results, with respect to the mis-targeting of IMPDH, were obtained when digitonin experiments with the ldpex14Δ 1-23-HA parasites were compared to wildtype parasites. Digitonin selectively permeabilizes the cholesterol-rich plasma membrane of cells at low detergent concentrations whereas higher concentrations are required to disrupt membranes of organelles like the glycosome. When ldpex14Δ 1-23-HA transgenic parasites were treated with increasing digitonin concentrations (0 mg/ml to 0.36 mg/ml), the glycosomal marker IMPDH was released early and is concurrent with the cytosolic enzyme APRT (Fig. 12, *panel* A). IMPDH is released only at higher concentrations of digitonin in wildtype promastigotes (Fig. 12, *panel* B).

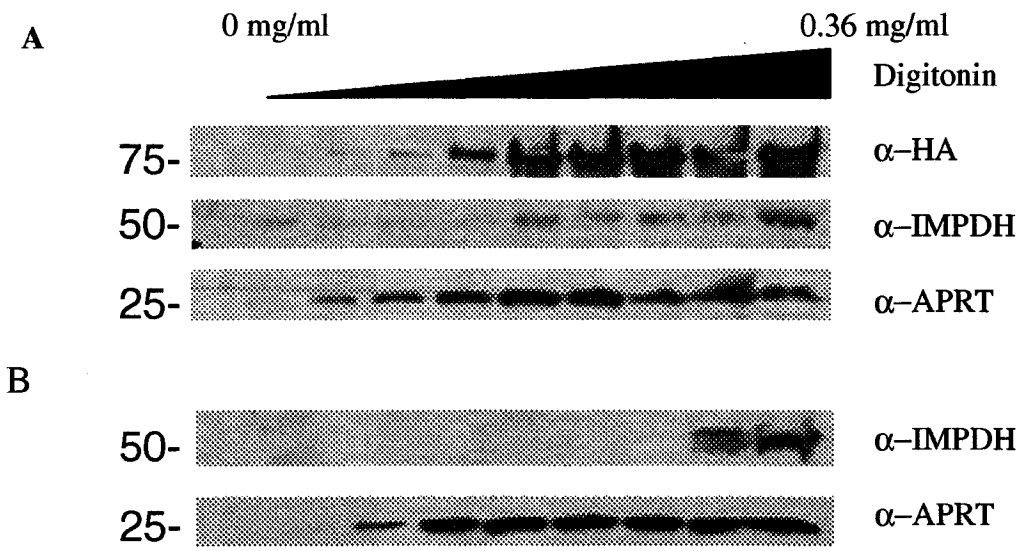

Figure 12. Digitonin permeabilization of ldpex14Δ 1-23-HA transgenic promastigotes. (A) Western blot analysis of the permeabilized promastigotes showed that ldpex14Δ 1-23-HA was released at lower concentrations of digitonin. Similar results were obtained for the cytosolic marker APRT. Interestingly, the glycosomal marker IMPDH also appears at lower digitonin concentrations indicating that IMPDH is slightly mistargeted due to the expression of ldpex14Δ 1-23-HA. (B) Digitonin permeabilization of wildtype *L .donovani* promastigotes showed that IMPDH is released only at higher digitonin concentrations.

The experiments taken together suggest that the expression of ldpex14Δ 1-23-HA is leading to the mis-targeting of the known glycosomal protein IMPDH (Dobie *et al.* 2007). The mis-targeting of IMPDH upon the introduction of ldpex14Δ 1-23-HA suggests that the ldpex14Δ 1-23-HA discovered in the cytosol of the parasite may be interacting with LdPEX5 and thereby leading to the mis-targeting of IMPDH to the cytosol. It is important to note that the binding of LdPEX5 to LdPEX14 has been narrowed to the N-terminal region between amino acids 23 and 63 (Madrid *et al.* 2005). Therefore the capacity for cargo-loaded PEX5 to bind to the ldpex14Δ 1-23-HA population found in the cytosol exists and may explain the mis-targeting of known glycosomal markers such as IMPDH, which contains a PTS-1 targeting signal.

LdPEX14 Oligomerization

Subcellular fractionation on ldpex14Δ 1-23-HA transgenic parasites revealed that the mutant protein did not co-sediment in the same fractions where the cytosolic protein APRT is found (Zarella-Boitz et al. 2004) (Fig. 11). ldpex14 Δ1-23-HA was detected in fractions 10–23 (Fig. 11) at 30%-55% sucrose concentrations. Cytosolic proteins such as APRT, a monomeric protein of approximately 27 kDa, were found to sediment at 20-25% sucrose concentrations (Fig. 11 *fractions 1 – 8*). This suggests that the ldpex14Δ 1-23-HA mutant protein could be forming oligomers of various densities resulting in different migration in the sucrose gradient. Previous studies have demonstrated that recombinant LdPEX14 is capable of forming homopolymers (Madrid *et al.* 2005). A single immunoreactive band corresponding

to ldpex14Δ 1-23-HA observed in fractions 10-19 and a doublet in fractions 20-24 that denote the presence of both native LdPEX14 and ldpex14 Δ1-23-HA associated with the glycosomal membrane suggest that ldpex14 Δ1-23-HA is oligomeric.

To evaluate if the ldpex14Δ 1-23-HA oligomerizes in a similar fashion to the native LdPEX14, subcellular fractionation experiments were performed on parasite preparations treated with 1% igepal (Fig. 13). Igepal is a non-detergent that will solubilize membrane-associated proteins, disrupting protein interactions, thus leaving protein complexes intact (Shetty *et al.* 2001). Western blot analysis of the sucrose gradient fractions of ldpex14Δ 1-23-HA 1% igepal-treated parasites showed that the HA signal localizes predominantly in fractions 10-17, indicating that the igepal did not alter the sedimentation of the free ldpex14Δ 1-23-HA. The HA signal previously observed in fractions 18-24 is much less pronounced indicating that the ldpex14Δ 1-23-HA associated with the glycosomes was solubilized. When the Western blots were probed with anti-LdPEX14, the doublet associated with the native and mutant ldpex14Δ 1-23-HA shifted to fractions 10-17 indicating that both ldpex14Δ 1-23-HA and native LdPEX14 co-sedimented at similar densities in the sucrose gradient (Fig. 13). These results suggest that ldpex14Δ 1-23-HA present in the cytosol is forming a homopolymeric structure similar to the native LdPEX14. Sucrose gradient fractions of igepal treated parasites probed with anti-IMPDH, revealed that IMPDH shifted from fractions 17-23 to fractions 9-12, verifying that the glycosomes were solubilized. IMPDH sedimented in fractions 9-12 due to its propensity to form a tetrameric structure of approximately 250 kDa. Finally to

confirm that the igepal had no effect on cytosolic proteins, samples were probed with anti-APRT which showed similar localization in both the untreated and 1% igepal-treated experiments from fractions 1 –8.

To demonstrate that native LdPEX14 formed oligomeric complexes that sediment in the 30-45% region of the sucrose gradient, wildtype cells were treated with igepal in a similar fashion to the ldpex14Δ 1-23-HA transgenic parasites. Subcellular fractionation of untreated wildtype cells revealed that the LdPEX14 and IMPDH both localized to fractions 15-21 corresponding to glycosomal localization (Fig. 13, *panel* B). Some IMPDH proteins were observed in fractions 1-5 however it is most likely due to glycosome disruption during hypotonic lysis. When wildtype cells were treated with 1% igepal prior to loading on the sucrose gradient, the localization of LdPEX14 and IMPDH showed altered migration from fractions 15-21 to fractions 7-15 (Fig. 13, *panel* C). The observed pattern suggested that the native LdPEX14 formed oligomeric structures that sediment in the 30-45% sucrose concentration.

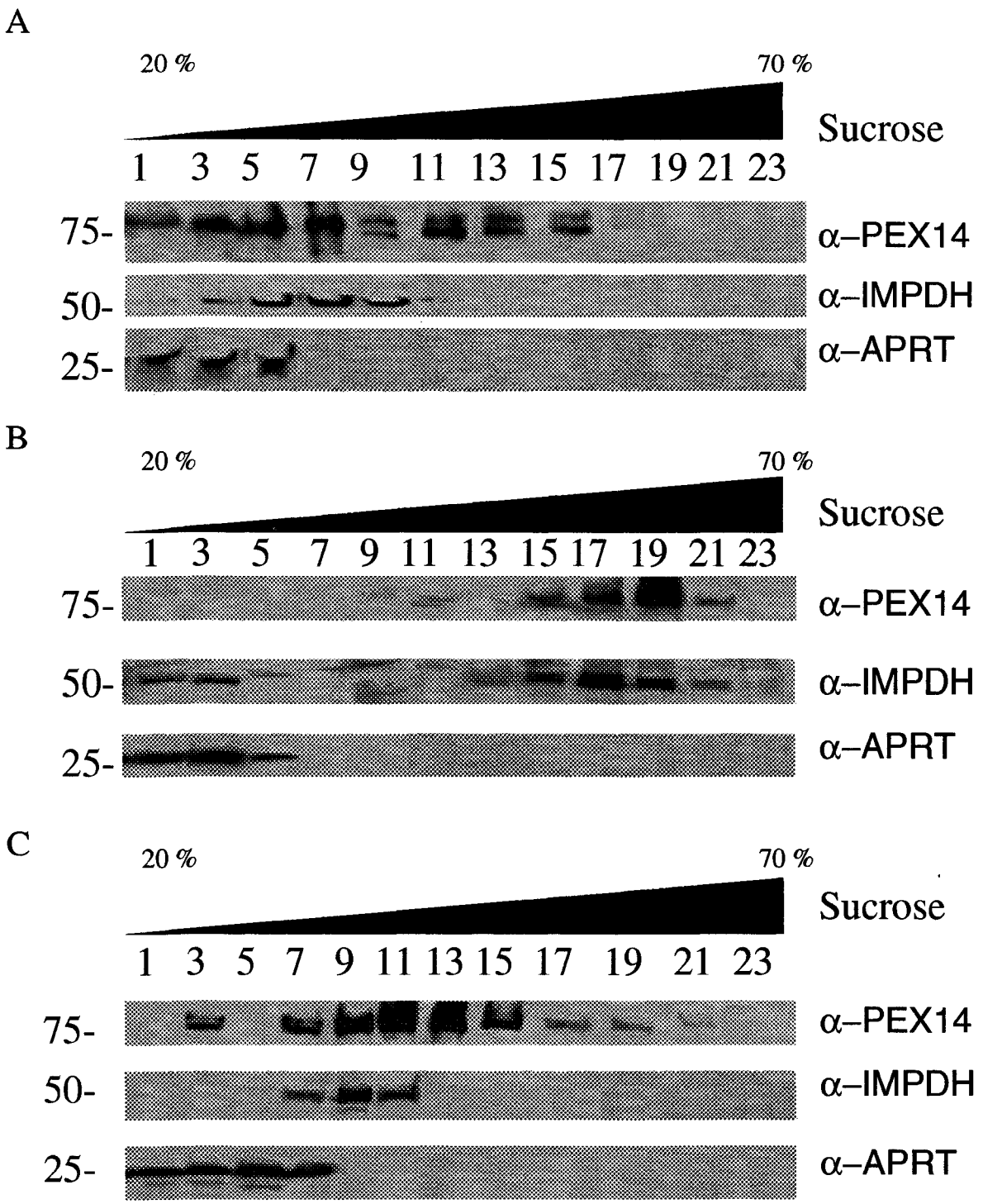

Figure 13. Igepal extraction experiments. (A) ldpex14Δ 1-23-HA transgenic parasites were extracted with 1% igepal and the supernatant was loaded onto a sucrose density gradient (20%-70%). (B) Subcellular fractionation of wildtype parasites and western blot analysis using anti-LdPEX14, anti-IMPDH and anti-APRT. (C) Wildtype parasites treated with 1% igepal and run on a linear sucrose gradient probed with anti-LdPEX14, anti-IMPDH and anti-APRT.

That LdPEX14 formed oligomeric complexes, was further assessed by analyzing purified recombinant His/S-LdPEX14-HA on a sucrose gradient. Coomassie blue stained SDS-PAGE gel (Fig. 14) revealed that recombinant His/S-LdPEX14-HA formed oligomeric structures that were recovered primarily in fractions 12 and 13 of the sucrose gradient. This sedimentation was similar to the native LdPEX14 and the ldpex14Δ 1-23-HA observed in *L. donovani* promastigotes. These results would suggest that the complex observed *in vivo* likely contains LdPEX14 subunits.

Understanding that LdPEX14 forms homopolymeric complexes it was important to evaluate the size of this complex. Using a known oligomeric complex that sediments in the sucrose gradient would allow us to speculate on the size of the LdPEX14 complex. The *Leishmania* proteasome is a complex of various proteins that is 20S in size (Paugam *et al.* 2003). To evaluate the size of the LdPEX14 complex, igepal extracts of wildtype parasites were run on a sucrose gradient and Western blots were probed with anti-human ubiquitin 20S proteasome antibodies, which cross-reacts with proteasome complexes from several different species, including *Leishmania.* The *Leishmania* 20S proteasome was detected in fractions 12 –17 of the gradient (Fig. 15). Previous results probing with anti-LdPEX14 revealed that the free native LdPEX14 and the ldpex14Δ 1-23 sediment at a similar density in the sucrose gradient (Fig. 15, *fractions* 9-17). This would suggest that native LdPEX14 and ldpex14Δ 1-23-HA form oligomeric complexes that are approximately 20S in size.

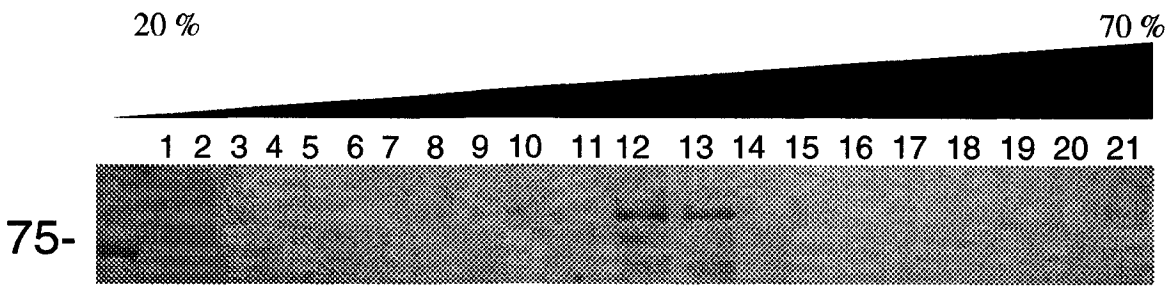

Figure 14. Sucrose density gradient on recombinant His/S-LdPEX14. The coomassie stained SDS-PAGE demonstrates that the recombinant His/S-LdPEX14 localizes in the 30 –45 % region on the sucrose gradient.

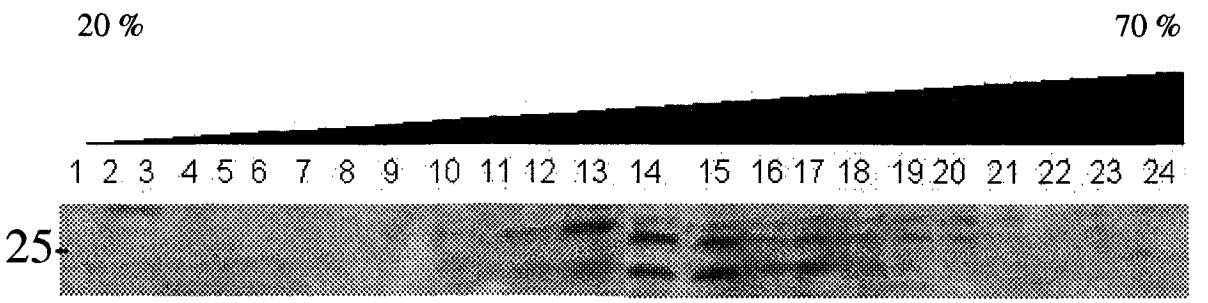

Figure 15. *Leishmania* proteasome subcellular localization. Wildtype parasites (1% igepal treated) run on a linear sucrose gradient and analyzed by western blot using anti-human ubiqutin 20S proteasome. The ubiquitin 20S proteasome can be found in the 30 – 45% region of the sucrose gradient. The anti-human ubiquitin 20S proteasome reacts with multiple proteins found in the proteasome complex.

FlAG-ldpex14 1-119 transgenic *L. donovani* parasites

To investigate that the N-terminal portion was sufficient for glycosomal targeting, *L. donovani* promastigotes were transfected with pALT-NEO FLAG-*ldpex*14 1-119 encoding the first 119 amino acids of LdPEX14 (Fig. 4). Expression of FLAG-ldpex14 1-119 in transgenic parasites resulted in an altered phenotype (Fig. 16). Parasites expressing FLAG-ldpex14 1-119 were round and stumpy when compared to wildtype or parasites expressing FLAG-ldpex14-HA. The FLAG-ldpex14 1-119 parasites measured approximately 17 microns in length whereas the full length FLAG-LdPEX14-HA transgenic parasites were approximately 25 microns.

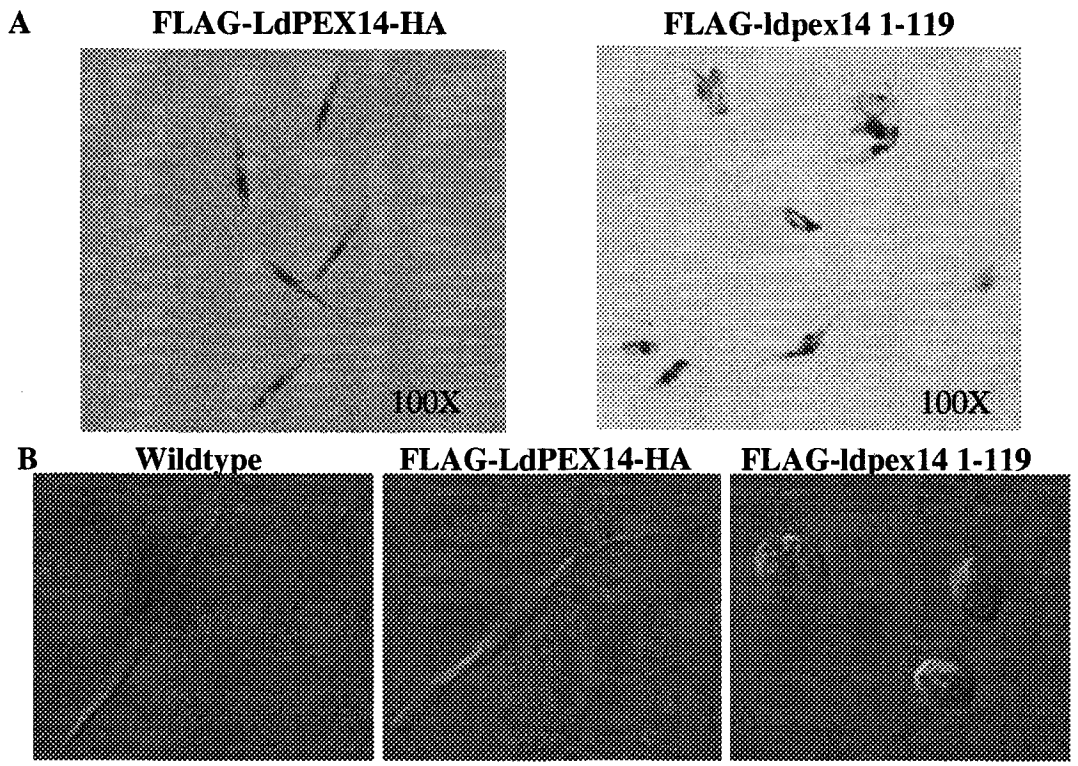

Figure 16. Cellular morphology of FLAG-ldpex14 1-119 transgenic parasites. Logphase transgenic parasites grown in 100 µg/ml G418. (A) Giemsa-stained parasites. The FLAG-LdPEX14-HA parasites demonstrate a normal morphology when compared to the irregular shape observed for the FLAG-ldpex14 1-119 transgenic parasites. (B) Images of fixed parasites.

Western blots of parasites expressing ldpex14 1-119 grown in media containing 20 µg/ml G418, probed with anti-LdPEX14 and anti-FLAG antibodies revealed no immunoreactive band corresponding to the FLAG-ldpex14 1-119 protein. It is important to note that although the level of FLAG-ldpex14 1-119 was below the detection limit, an altered phenotype was still evident. At higher drug concentrations of 100 and 200 µg/ml G418, a 25 kDa band was detected during western blot analysis using anti-LdPEX14 antibodies. No band was present in wildtype or FLAG-LdPEX14-HA parasites (Fig. 17). No band was detected when experiments were performed using anti-FLAG antibodies. An interesting observation was that parasites expressing FLAG-ldpex14 1-119 appeared to have a reduced level of native LdPEX14. It is possible that the amount of native LdPEX14 protein is down-regulated due to the presence of the toxic FLAG-ldpex14 1-119.

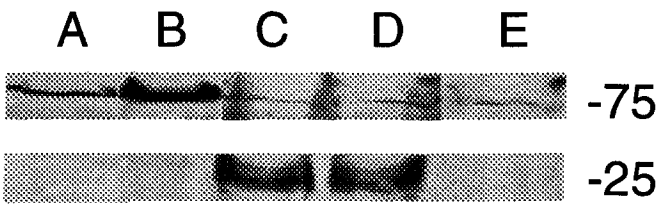

Figure 17. Expression of ldpex14 fragments in *L. donovoani* parasites. 1 x 10^7 cells/ml run on SDS-PAGE analyzed by Western Blot using anti-LdPEX14. (A) Wildtype cells and (B) FLAG-LdPEX14-HA transgenic promastigotes show no band at 25 kDa. (C) FLAG-ldpex14 1-119 transgenic parasites grown at 200 µg/ml G418 and (D) 100 µg/ml G418 show a band indicative of the expression of FLAG-ldpex14 1-119 at 25 kDa. (E) No band is observed for the FLAG-ldpex14 1-119 transgenic parasites grown at 20 µg/ml G418.

Attempts to localize FLAG-ldpex14 1-119 by confocal immunofluorescence microscopy was difficult to monitor due to the lack of a detectable epitope tag. Western blot analysis of subcellular fractionation samples of parasites expressing FLAG-ldpex14 1-119 grown in 100 μg/ml G418 revealed an anti-LdPEX14 immunoreactive band at 25 kDa in the organellar fraction which is typically rich in glycosomes (Fig. 18). Similar experiments performed on FLAG-LdPEX14-HA transgenic parasites grown in 100 μg/ml G418 or wildtype parasites showed no similar band in the cytosol or organellar fractions. All three parasite strains exhibited faithful localization of the native LdPEX14 to the glycosomal fraction, identifiable by the band at 75 kDa. The localization of FLAG-ldpex14 1-119 to the glycosomal membrane indicates that the N-terminal region of LdPEX14 is sufficient for binding to the glycosomal membrane.

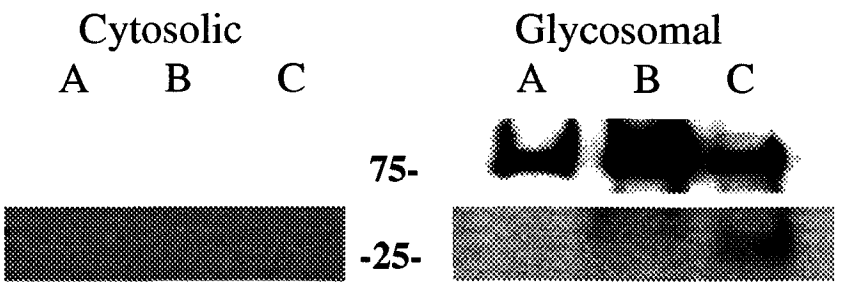

Figure 18. Cellular fractionation of FLAG-ldpex14 1-119 *L. donovani* parasites run on a 30% Nycodenz gradient probed with anti-LdPEX14. (A) Wildtype parasites (B) FLAG-LdPEX14-HA transgenic parasites and (C) FLAG-ldpex14 1-119 transgenic parasites

Growth kinetics of FLAG-ldpex14 1-119 transfectants

To further evaluate the toxic effects of FLAG-ldpex14 1-119, growth curves comparing wildtype parasites, FLAG-LdPEX14-HA transgenic parasites and FLAG-ldpex14 1-119 transgenic parasites were performed. The three parasites strains were seeded at 5 x 10^5 cells/ml and the growth was monitored by counting every second day. The wildtype cells and FLAG-LdPEX14-HA transgenic cells, the latter grown at 50 μg/ml G418, exhibited normal growth kinetics (Fig. 19). The parasites demonstrated a lag phase of one day before increasing their proliferation rate during log phase. The wildtype and FLAG-LdPEX14-HA transgenic parasites reached stationary phase at day 8 and day 9, respectively. The final density reached by both the wildtype and FLAG-LdPEX14-HA parasites was approximately 4 x 10^7 cells/ml. When the growth of the FLAG-ldpex14 1-119 transgenic parasites was evaluated, a difference in growth patterns was observed when compared to the wildtype and FLAG-LdPEX14-HA growth curves (Fig. 19). The FLAG-ldpex14 1-119 transgenic parasites grown at 50 μg/ml G418 exhibited a longer lag phase of 3 days. Furthermore, the proliferation/survival of the FLAG-ldpex14 1-119 was severely attenuated reaching stationary phase at day 9 and reached a low cell density of approximately 2 x 10^7 cells/ml. The difference in growth patterns in the FLAG-ldpex14 1-119 transfectants further suggests a toxic effect from expression of the FLAG-ldpex14 1-119 protein.

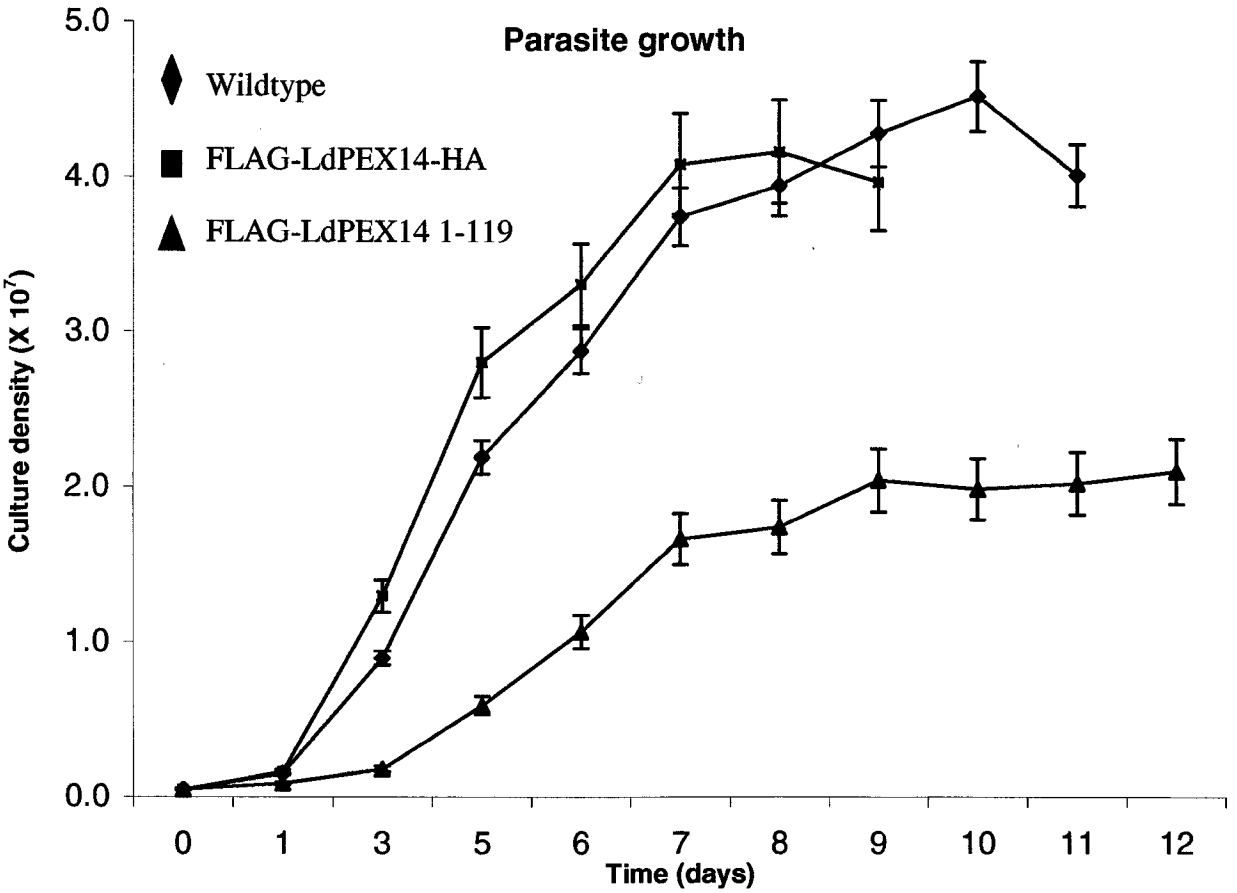

Figure 19. Growth curve of FLAG-ldpex14 1-119 transgenic parasites, FLAG-LdPEX14-HA transgenic parasites and wildtype *Leishmania donovani* promastigotes. The FLAG-ldpex14 1-119 transgenic parasites showed a reduced ability to grow under similar conditions as the wildtype and FLAG-LdPEX14-HA strains.

The FLAG-ldpex14 1-119 transgenic parasites have shown a reduced ability to proliferate/survive due to a possible toxic effect. One final observation regarding the phenotypic changes associated with the introduction of FLAG-ldpex14 1-119 was the reduced motility and tendency of these parasites to move in a circular pattern. In culture, the FLAG-ldpex14 1-119 transgenic parasites sedimented to the bottom of the culture flask, whereas wildtype *L. donovani* promastigotes and FLAG-LdPEX14-HA transgenic parasites grown in culture move freely in the media with little sedimentation at the bottom of the flask.

Metabolic consequences of FLAG-ldpex14 1-119 expression

T. brucei experiments involving RNAi disruption of TbPEX14 have demonstrated that the lack of PEX14 results in the mis-targeting of known glycosomal markers to the cytosolic compartment (Moyersoen *et al.* 2004; Moyersoen *et al.* 2003; Furuya *et al.* 2002). The loss of TbPEX14 ultimately results in the death of the parasites as several important metabolic functions are disrupted. To evaluate if a similar event is occurring in the FLAG-ldpex14 1-119 transgenic parasites, confocal immunofluorescence microscopic analysis was performed to determine the localization of IMPDH. When parasites were probed with anti-IMPDH and visualized with anti-guinea-pig FITC, a punctate staining pattern was observed indicative of glycosomal localization (Fig. 20, *panel* A). When FLAG-ldpex14 1-119 transgenic parasites were probed with anti-LdPEX14, a punctate staining pattern was also observed (Fig 20, *panel* B). When the images are overlaid co-localization

is observed indicating that both IMPDH and PEX14 are localized to the glycosome in the FLAG-ldpex14 1-119 transgenic parasites. Experiments performed on wildtype promastigotes yielded a similar staining pattern (Fig. 20, *panel* E,F,G,H), indicating that the localization of IMPDH is unaltered in the FLAG-ldpex14 1-119 transgenic parasites

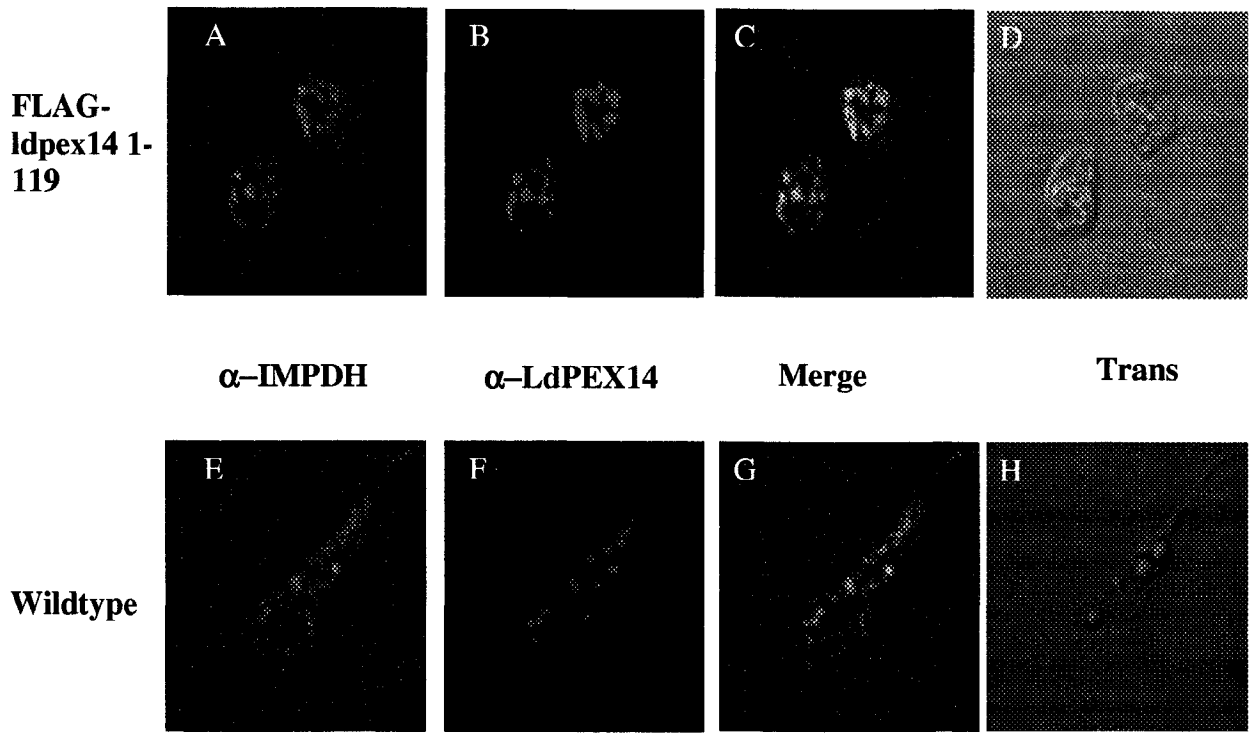

Figure 20. Confocal immunofluorescence microscopy of FLAG-ldpex14 1-119 transgenic promastigotes. (A) Signal representing anti-IMPDH visualized with anti-guinea pig FITC demonstrates glycosomal localization. (B) Signal from anti-LdPEX14 antibodies visualized with an anti-rabbit Cy3 antibody demonstrates glycosomal staining. The merged image (C) showed that the two signals co-localize indicating that the localization of IMPDH and LdPEX14 are unaltered in the FLAG-ldpex14 1-119 transgenic promastigotes. Confocal immunofluorescence microscopy on wildtype promastigotes were used as a control. (E) Signal representing anti-IMPDH visualized with anti-guinea pig FITC demonstrates glycosomal localization. (E) Signal demonstrated from anti-LdPEX14 antibodies visualized with an anti-rabbit Cy3 antibody. (F) Merged image demonstrates co-localization of the two signals.

To evaluate the localization of glycosomal proteins in FLAG-ldpex14 1-119 transgenic parasites, digitonin permeabilizations were performed on the parasites. Western blot analysis revealed that IMPDH and XPRT were released at the two highest digitonin concentrations (Fig. 21). The signals for IMPDH and XPRT did not co-localize with the known cytosolic protein APRT. When similar concentrations of digitonin were added to wildtype cells, IMPDH and XPRT also appeared at the two highest concentrations. The confocal immunofluorescence microscopic analysis and digitonin experiments performed on FLAG-ldpex14 1-119 suggest that the glycosomal proteins are being properly targeted to the glycosome.

The final analysis performed on the FLAG-ldpex14 1-119 was an alamar blue assay to assess the reducing potential of the FLAG-ldpex14 1-119 transgenic parasites. The alamar blue dye is reduced from a blue non-fluorescent dye to a pink fluorescent dye in the presence of live parasites. If there is a difference in metabolic activity in the FLAG-ldpex14 1-119, an altered fluorescent pattern should be observed when incubated with alamar blue and compared to healthy parasite strains. When FLAG-ldpex14 1-119 (100 μg/ml G418), FLAG-LdPEX14-HA transgenic parasites (100 μg/ml G418) and wildtype parasites were incubated with alamar blue, no difference was observed with respect to the reduction of alamar blue for the three strains after 24 hrs. All three strains showed similar fluorescene emittance of approximately 1, 000, 000 fluorescent units. This would suggest that the FLAG-ldpex14 1-119 parasites are viable as they are capable of reducing the alamar blue similarly to the FLAG-LdPEX14-HA transgenic parasites.

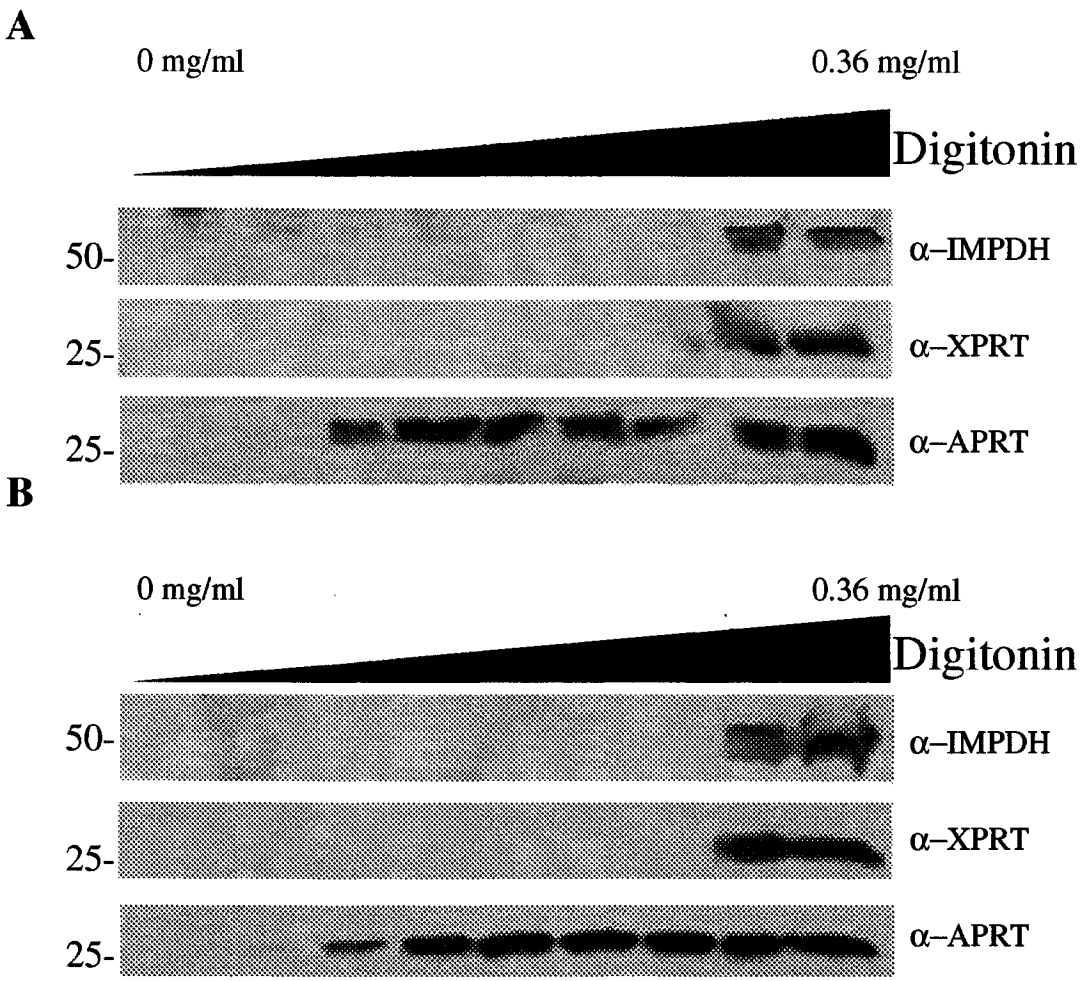

Figure 21. Digitonin permeabilization of FLAG-ldpex14 1-119 transgenic promastigotes. (A) The signal observed for known glycosomal markers IMPDH and XPRT is unaltered when comparing FLAG-ldpex14 1-119 transgenic parasites to (B) wildtype parasites treated under similar digitonin concentrations (0 mg/ml to 0.36 mg/ml).

Localization of FLAG-ldpex14 1-74-GFP

To demonstrate that the N-terminal region of LdPEX14 was sufficient for interacting with the glycosomal membrane, a construct consisting of the first 74 amino acids of LdPEX14 was created, FLAG-ldpex14 1-74-GFP (Fig. 4). Confocal immunofluorescence microscopic analysis performed at 10 days post-transfection demonstrated a punctate staining pattern for GFP, indicative of glycosomal localization (Fig. 22, *panel* A). When parasites were probed with anti-LdPEX14 the signal co-localizes with the GFP signal. As a control, parasites transfected with pALT-NEO GFP-AKL were analyzed using confocal immunofluorescence microscopy. The staining revealed a similar co-localization of the GFP and anti-LdPEX14 signals (Fig. 22, *panel* B). Unexpectedly, at 40 days post-transfection, FLAG-ldpex14 1-74-GFP was observed mainly in the cytosolic compartment (Fig. 22, *panel* C). Similarly, when the late transfectants were probed with anti-FLAG the signal observed was in the cytosolic area. Interestingly, when the signals were merged there is no co-localization.

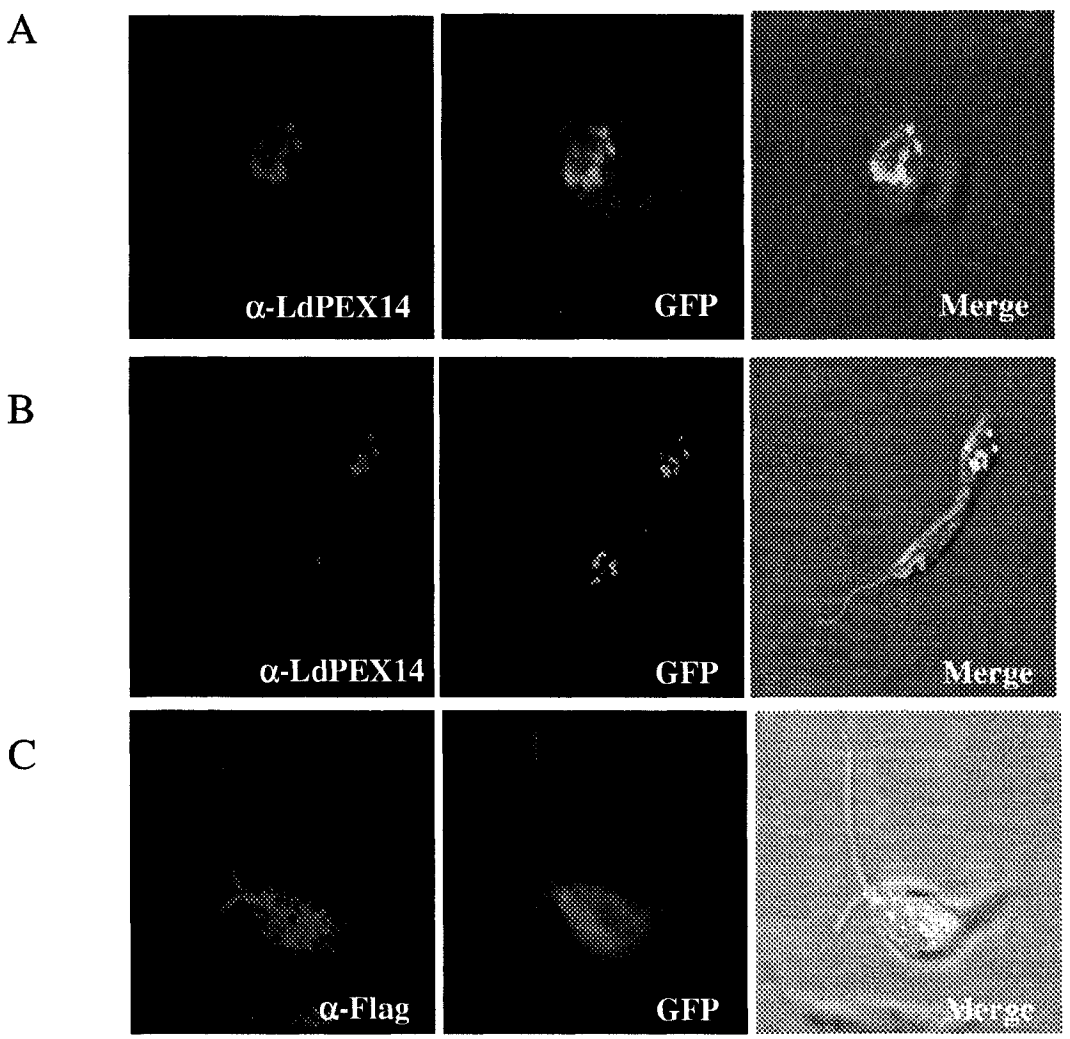

Figure 22. Confocal immunofluorescence microscopy on FLAG-ldpex14 1-74-GFP transgenic parasites 10 day post-transfection (A) showing punctate staining from GFP fluorescence and after staining with anti-LdPEX14 antibody and anti-rabbit Cy3. (B) pALT-NEO-GFP-AKL transgenic parasites expressing GFP showing punctate staining. The red signal represents anti-LdPEX14 and anti-rabbit cy3. (C) At 40 days post-transfection, FLAG-ldpex14 1-74 GFP transfectants show that the green signal, GFP, is localized to the cytosol. The red signal is anti-FLAG visualized with anti-rabbit Cy3.

To evaluate the discrepancy in the FLAG-ldpex14 1-74-GFP localization, subcellular fractionation experiments were performed on transfected promastigotes at the later timepoint as sufficient cell numbers were available (Fig. 23). When samples were probed with anti-FLAG antibodies, a single band was observed at approximately 28 kDa. The anti-FLAG signal observed was found to be in the cytosolic region of the gradient (*fractions* A-C). When samples were probed with anti-GFP antibodies, two bands are observed one at ~28 kDa and one at ~22 kDa. Similar to the anti-FLAG signal, the anti-GFP signal was only detected in the cytosolic region of the gradient. Only the ~28 kDa species was found to react with both the anti-FLAG and anti-GFP antibodies. The lower band reacted only with anti-GFP. Fractions probed with anti-XPRT, clearly showing that the GFP and FLAG signals are not localized to the glycosomal region at later timepoints. These results indicate that at late time points, the recombinant FLAG-ldpex14 1-74-GFP was degraded from the N-terminus or was cleaved at a region before the GFP. It is unknown why this cleavage/degradation is occurring.

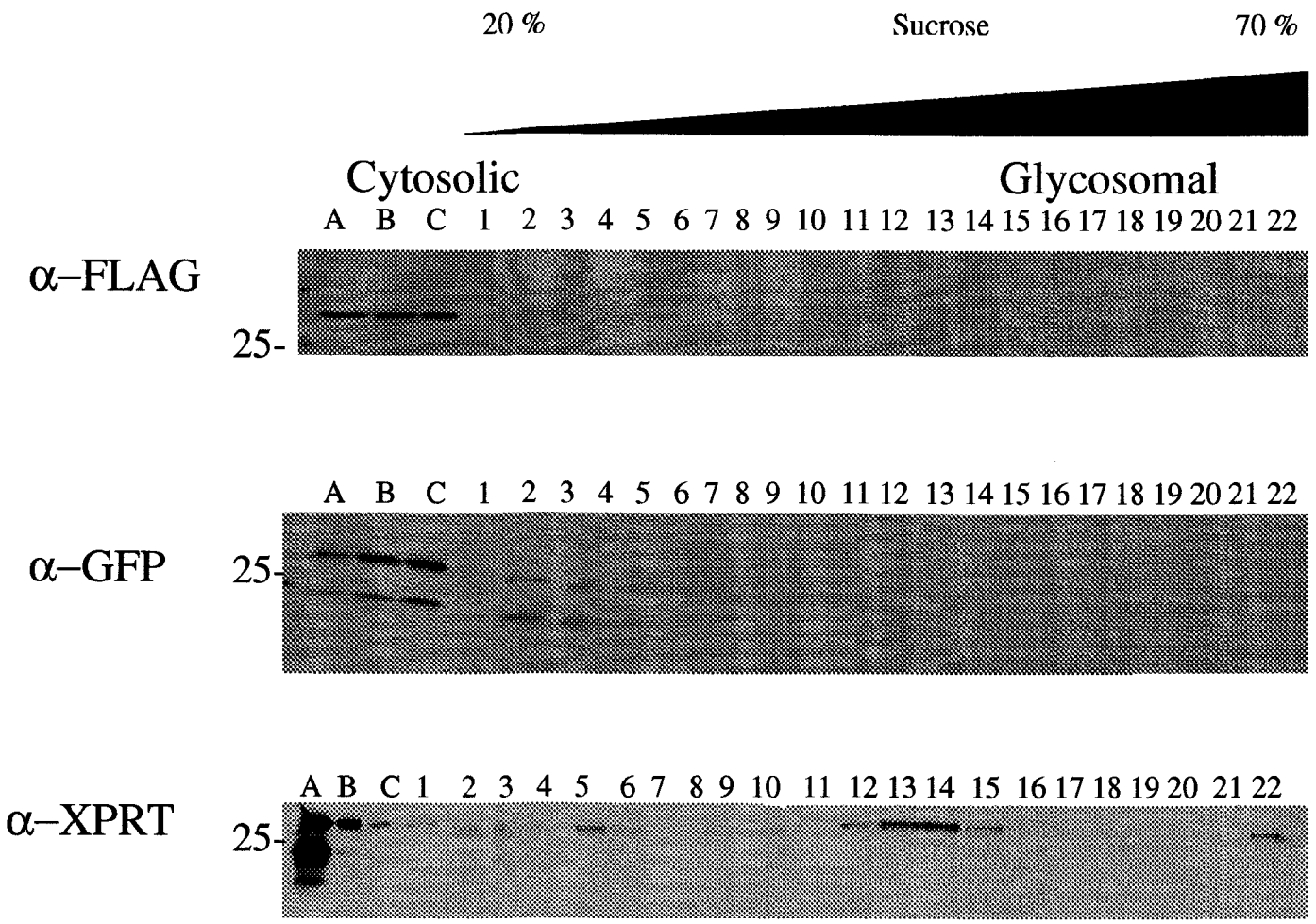

Figure 23. Sucrose density gradient fractionation of FLAG-ldpex14 1-74-GFP transgenic promastigotes 60 days post-transfection. (A: 3, 000 rpm pellet, B: 3, 000 rpm supernatant, C: 40, 000 x g supernatant, 1-22 Sucrose fractions) The anti-FLAG antibodies recognized a 25 kDa representing the FLAG-ldpex14 1-74-GFP protein found in the cytosolic fractions. Anti-GFP antibodies detected two bands at ~22 and ~28 kDa in the cytosolic fractions. The ~28 kDa band represents a protein encompassing the entire FLAG-ldpex14 1-74-GFP. The lower band represents solely the cleaved GFP population lacking the N-terminus region. The glycosomal marker XPRT is used as a control (bottom panel).

VI. DISCUSSION

The trypanosomatid glycosome performs several fundamental tasks which have been shown to be essential for the survival of the parasites. These vital processes include purine salvage, glycolysis, gluconeogenesis, pyrimidine and ether-lipid biosynthesis and β-oxidation of fatty acids (Opperdoes, 1987; Fairlamb, 1989; Rodriguez and Landfear, 2006). In order for these processes to occur within the glycosome, proteins must be imported using the PTS import pathway which involves a membrane receptor LdPEX14 (Jardim *et al.* 2002). LdPEX14 is quite unique from its homologues in other species such as humans and yeast. In mammalian (Shimizu *et al.* 1999), *H. polymorpha* (Komori *et al.* 1997; Komori *et al.* 1999), *Arabidopsis* (Hayashi *et al.* 2000), and *Pichia* (Johnson *et al.* 2001) cells, PEX14 appears to be an integral membrane protein that has either the C-terminus or both the N- and C-termini oriented toward the cytosolic compartment. In the case of LdPEX14, it has been shown to be a peripheral membrane protein which contains both the N and C-termini exposed to the cytosol (Jardim *et al.* 2002). The function of LdPEX14 is suggested to be similar to the other PEX14 proteins as it acts as a docking protein for the PEX5-PTS-1 complex. Therefore, the fact that LdPEX14 is a peripheral membrane protein, whereas the other PEX14 proteins are integral membrane proteins, makes the possible mechanism of import quite unique.

Since LdPEX14 is unique it was important to evaluate the regions involved in binding to the glycosomal membrane. Experiments with both the LdPEX14 N-terminal mutants, ldpex14Δ 1-63-HA and ldpex14Δ 1–23–HA, demonstrated that

the N-terminus is required for proper targeting to the glycosomal membrane, as removal of the N-terminal regions resulted in the accumulation of the recombinant protein in the cytosol of the parasite. Similar experiments performed with ScPEX14 demonstrated that the removal of the first 58 amino acids did not alter the localization of ScPEX14. However, removal of the truncated ScPEX14 from the peroxisomal membrane was facilitated suggesting that the N-terminal region significantly contributes to the insertion of ScPEX14 to the peroxisomal membrane (Niederhoff *et al.* 2005). This would suggest that in both the ScPEX14 as well as the LdPEX14, the extreme N-terminus is important for the efficient binding to the peroxisomal/glycosomal membrane.

The suggestion that the N-terminus of LdPEX14 was important for binding to the glycosomal membrane was confirmed by examining the localization of FLAG-ldpex14 1-74-GFP which showed targeting to the glycosomal membrane. This indicates that the presence of the first 74 amino acids of LdPEX14 is sufficient to bind the protein to the glycosomal membrane. Unexpectedly, FLAG-ldpex14 1-74-GFP transfectants examined at 40 days post-transfection exhibited both the GFP and FLAG signals localized to the cytosolic compartment (Fig. 22, *panel* C). It is possible that the FLAG-ldpex14 1-74GFP is toxic and that a subpopulation containing a rearranged construct of the FLAG-ldpex14 1-74-GFP emerged over time. Thus, the signal for both the FLAG epitope and the GFP signal are observed in the cytosol at late timepoints. It is also plausible that cleavage of the FLAG-

ldpex14 1-74-GFP has occurred resulting in the localization of both the GFP and the FLAG epitope in the cytosolic compartment.

The N-terminus of other PEX14 homologues contains a hydrophobic trans-membrane domain. Earlier studies have indeed demonstrated that LdPEX14 is a peripheral membrane protein as extraction with Triton X-114 removes the protein from the surface of the glycosomal membrane (Jardim *et al.* 2002). Therefore there are two suggested mechanisms of binding of LdPEX14 to the glycosomal membrane. The first involves the interaction of LdPEX14 with another protein found attached to the glycosomal membrane. When analyzing the N-terminal region of LdPEX14 from amino acids 1 to 40, it is rich in proline, alanine and glutamine residues which have a tendency to form a structure called a polyproline II helix (Rath *et al.* 2005). Polyproline II helices have been demonstrated to mediate protein-protein interactions, therefore if another protein contains a polyproline II helix it could mediate the binding of LdPEX14 to the glycosomal membrane. To date, extensive searches have been performed in order to identify interactive partners for LdPEX14, however none have been elucidated. Further proteomic-based studies should be done to find novel interacting proteins.

A second possibility includes the direct interaction of LdPEX14 with the glycosomal membrane. The N-terminal region of LdPEX14 contains several amphipathic residues that could account for the direct interaction with the lipid bilayer of the glycosome (Kogan *et al.* 2001). It is also possible that the presence of seven acidic

residues, which present a negative charge, are involved in the interaction with the phospholipid head groups of the glycosomal membrane (Quinones *et al.* 2004). Experiments have been undertaken to examine the nature of the LdPEX14 protein with liposomes. If LdPEX14 directly associates with the phospholipid head groups, attachment to liposomes should be observed *in vitro.*

The introduction of LdPEX14 mutants had some differing effects on the transgenic parasites. ldpex14Δ 1-23-HA lead to the mis-targeting of IMPDH to the cytosol of the parasite, however no phenotypic or toxic effects were observed due to the mis-targeting. The presence of the native LdPEX14 likely accounts for the ability of the parasite to survive normally even though the IMPDH localization is altered. The mis-targeting of IMPDH and other PTS-1 proteins is likely due to the accumulation of ldpex14Δ 1-23-HA in the cytosolic compartment of the parasites. The ldpex14Δ 1-23-HA retains the LdPEX5 binding domain between amino acids 23 and 63 allowing the cytosolic ldpex14Δ 1-23-HA to interact with LdPEX5 thus altering the targeting of known glycosomal proteins (Fig. 24. *panel* B). Similar experiments performed on yeast pex14 knockouts complemented with ScPEX14Δ 1-58 demonstrated an altered targeting with respect to both PTS1 and PTS2 peroxisomal proteins (Niederhoff *et al.* 2005). However, the ScPEX14Δ 1-58, unlike the ldpex14Δ 1-23-HA, was faithfully targeted to the peroxisome.

An interesting observation from the sucrose gradients performed on the ldpex14Δ 1-23-HA transgenic parasites was the accumulation of ldpex14Δ 1-23-HA in the

cytosolic compartment. Through igepal extraction experiments the ldpex14Δ 1-23-HA was shown to be oligomeric in nature of comparable size to the native oligomeric LdPEX14. These results suggest that ldpex14Δ 1-23-HA is forming a similar oligomeric structure as the native LdPEX14, even though it is localized to the cytosol. Two possibilities could account for the formation of the oligomeric ldpex14Δ 1-23-HA complex. It is known that LdPEX14 is capable of homopolymerization, therefore the spontaneous homopolymerization of ldpex14Δ 1-23-HA in the cytosol is plausible (Fig. 24, *panel* B). The second possibility includes the prospect that the ldpex14Δ 1-23-HA is forming oligomers by piggy-backing onto native LdPEX14 attached to the glycosome and then is ultimately released due to inefficient and weaker binding to the glycosomal membrane (Fig. 24, *panel* C). This would explain the oligomeric nature of the dual population of ldpex14Δ 1-23-HA, some attached to the membrane via native LdPEX14 with the remainder of the ldpex14Δ 1-23-HA released back to the cytosol. Experiments using recombinant LdPEX14 expressed in *E. coli* cells established that the recombinant LdPEX14 forms oligomeric complexes of similar density as both the native LdPEX14 and the ldpex14Δ 1-23-HA. The interaction of recombinant LdPEX14 occurs without the presence of glycosomes, suggesting that the ldpex14Δ 1-23-HA is forming oligomeric complexes without assistance from other proteins.

Both the native LdPEX14 and the ldpex14Δ 1-23-HA formed oligomeric complexes that sedimented in the 30-45% region of the sucrose gradient following igepal extraction. To evaluate the size of the LdPEX14 oligomeric structure it was

important to evaluate its sedimentation in reference to a known complex within the *Leishmania* parasite. The 20S proteasome is a complex of several proteins that has been studied in *Leishmania* (Paugam *et al.* 2003). The 20S proteasome was found to sediment in the 30-45% sucrose range indicating that the LdPEX14 oligomeric structures were of similar size to the 20S proteasome. The confirmation and components of the LdPEX14 and ldpex14Δ 1-23-HA oligomer remains to be confirmed and experiments have been undertaken to visualize the confirmation of the protein on the glycosomal membrane.

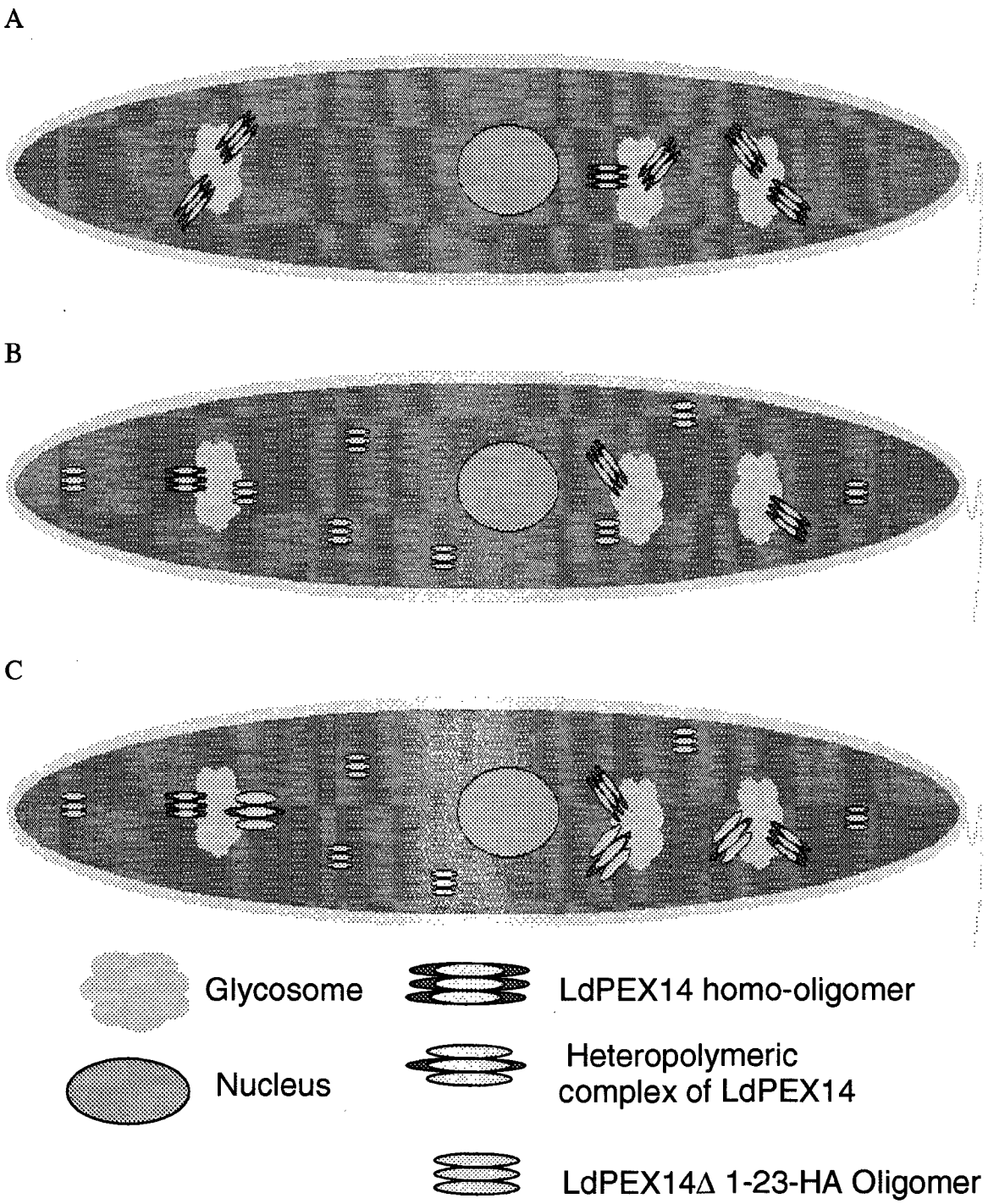

Figure 24. Schematic representation of ldpex14Δ 1-23-HA localization. (A) Wildtype parasites showing the localization of the oligomeric structure of native LdPEX14. (B) Transgenic parasites with ldpex14Δ 1-23-HA localized both in the cytosol and attached to the glycosomal membrane. (C) ldpex14Δ 1-23-HA in the cytosol with a population interacting and forming oligomers with the native LdPEX14 on the glycosomal membrane.

The second LdPEX14 mutant that resulted in an altered phenotype was the FLAG-ldpex14 1-119, which provided an altered morphology of the parasites and a stunted growth pattern indicating that the lack of the C-terminus confers a toxic effect to the protein. Interestingly, the localization of known glycosomal proteins, IMPDH and XPRT was unaltered, indicating that the phenotype observed in the FLAG-ldpex14 1-119 was not due to the accumulation of glycosomal proteins in the cytosol. Furthermore, the localization of FLAG-ldpex14 1-119 was demonstrated to be glycosomal. FLAG-ldpex14 1-119 has been shown to make dimers *in vitro,* it is possible that the FLAG-ldpex14 1-119 is binding to the glycosomal membrane and preventing the functional import of proteins into the glycosome. In this case the interaction of FLAG-ldpex14-119 with the LdPEX5-PTS1 complex would not lead to the incorporation of the PTS-1 protein. However, it would remain on the glycosomal membrane (Fig. 25). Experiments with yeast showed that the removal of the C-terminal residues of ScPEX14 results in a *pex* phenotype, identifiable by residual, ghost-like peroxisomes resulting in the accumulation of peroxisomal proteins in the cytosol (Niederhoff *et al.* 2005). In the case of the FLAG-ldpex14 1-119, no accumulation of glycosomal proteins are observed in the cytosol. Experiments have been undertaken to further examine the mechanism at play with respect to the FLAG-ldpex14 1-119. If the LdPEX5-PTS1 complex is being trapped on the glycosomal membrane, there should be an evident alteration in the localization of LdPEX5, a normally cytosolic protein.

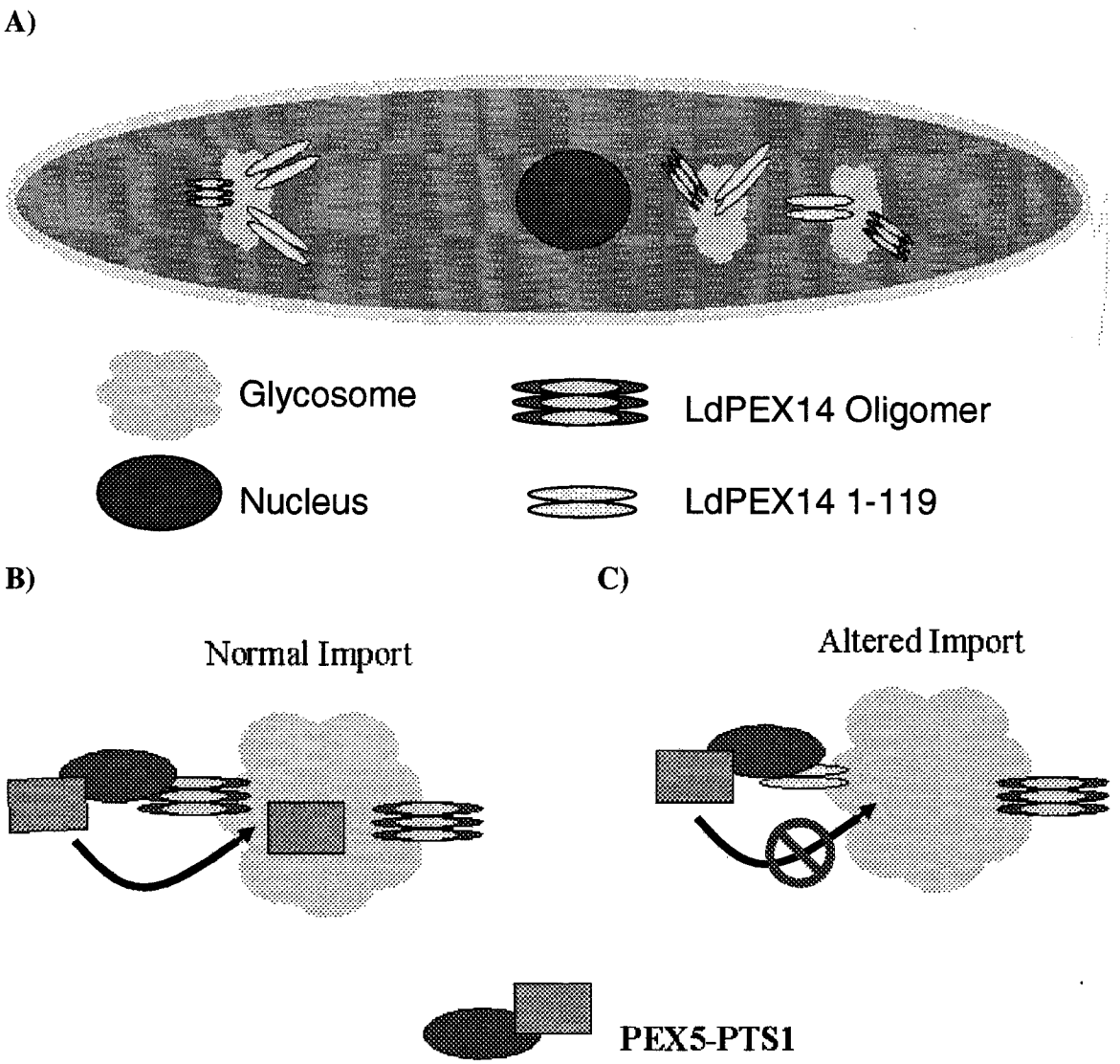

Figure 25. (A) Schematic representation of the localization of the dimeric FLAG-ldpex14 1-119 to the glycosomal membrane. (B) Normal import of PTS1 proteins showing import upon interaction of LdPEX5 with native LdPEX14. (C) Altered import showing the inhibition of import, due to some unknown mechanism, possibly the binding of LdPEX5 to the dimeric FLAG-ldpex14 1-119 on the glycosomal membrane.

LdPEX14 is a unique protein that has been shown to exhibit different properties in comparison to other PEX14 homologues. Other homologues of PEX14 have been shown to be integral membrane proteins, whereas LdPEX14 is a peripheral membrane protein. Despite the different properties of the PEX14 homologues it appears that the N-terminus of LdPEX14 is still important for its association to the glycosomal membrane. Further studies are required to understand the topology of the LdPEX14 oligomer on the glycosomal membrane. Is it interacting with other PEX proteins or is it interacting directly with the phospholipds of the membrane. LdPEX14 is at the center of protein import into the glycosome, and thus remains an important protein for further understanding the complex interactions that take place at the glycosomal membrane.

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