Biochemical and molecular characterization of the glycosomal PTS2 import receptor peroxin 7 in *Leishmania donovani*

By

Ana Victoria Pilar

Institute of Parasitology, McGill University, Montreal

February 2009

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Science

© 2009 Ana Victoria C. Pilar
Table of contents

Table of contents........................................................................................................... ii
Abstract...................................................................................................................... viii
Abrégé ......................................................................................................................... ix
Contribution of authors............................................................................................... x
Claims of originality ................................................................................................. xi
Abbreviations used ................................................................................................. xii
Acknowledgements ................................................................................................. xiv
List of tables.............................................................................................................. xvii
List of figures .......................................................................................................... xvii
CHAPTER 1. GENERAL INTRODUCTION ..............................................................1
Thesis Objectives .......................................................................................................4
Chapter 1 References ...............................................................................................5
CHAPTER 2. LITERATURE REVIEW ......................................................................10
Leishmania taxonomy ..............................................................................................10
  Taxonomic classification .......................................................................................10
  Characteristics of Kinetoplastids ......................................................................11
  Evolution of Kinetoplastids ..............................................................................11
Leishmania biology .................................................................................................13
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life cycle</td>
<td>13</td>
</tr>
<tr>
<td>Medical importance of trypanosomatids</td>
<td>15</td>
</tr>
<tr>
<td>Diseases caused by <em>Leishmania</em> sp.</td>
<td>15</td>
</tr>
<tr>
<td><em>Leishmania</em>-HIV co-infection</td>
<td>17</td>
</tr>
<tr>
<td>Diseases caused by <em>Trypanosoma</em> spp.</td>
<td>18</td>
</tr>
<tr>
<td>Factors involved in the transmission and pathology of leishmaniasis</td>
<td>19</td>
</tr>
<tr>
<td>Parasite-vector interactions</td>
<td>19</td>
</tr>
<tr>
<td>Host-parasite interactions</td>
<td>20</td>
</tr>
<tr>
<td>Diagnosis of leishmaniasian</td>
<td>22</td>
</tr>
<tr>
<td>Serological tools</td>
<td>23</td>
</tr>
<tr>
<td>Molecular diagnostic tools</td>
<td>24</td>
</tr>
<tr>
<td>Diagnosis of HIV/<em>Leishmania</em> co-infections</td>
<td>25</td>
</tr>
<tr>
<td>Summary statement</td>
<td>25</td>
</tr>
<tr>
<td>Treatment of leishmaniasian</td>
<td>26</td>
</tr>
<tr>
<td>Summary statement</td>
<td>29</td>
</tr>
<tr>
<td>Mechanism of drug resistance</td>
<td>29</td>
</tr>
<tr>
<td>Vaccine development</td>
<td>30</td>
</tr>
<tr>
<td>Summary statement</td>
<td>32</td>
</tr>
<tr>
<td>Vector and reservoir control</td>
<td>33</td>
</tr>
<tr>
<td>Summary statement</td>
<td>34</td>
</tr>
<tr>
<td>Glycosome biology</td>
<td>34</td>
</tr>
<tr>
<td>General features of glycosomes</td>
<td>35</td>
</tr>
<tr>
<td>Enzymatic content of glycosomes</td>
<td>36</td>
</tr>
<tr>
<td>Advantages of glycosomes and glycolytic compartmentation</td>
<td>38</td>
</tr>
<tr>
<td>Biogenesis of glycosomes</td>
<td>39</td>
</tr>
<tr>
<td>PEX proteins</td>
<td>40</td>
</tr>
<tr>
<td>Glycosomal and peroxisomal import signals</td>
<td>41</td>
</tr>
</tbody>
</table>
CHAPTER 3. INTERACTION OF LEISHMANIA PTS2 RECEPTOR Peroxin 7 WITH THE GLYCOosomal PROTEIN IMPORT MACHINERY

Abstract

Introduction

Materials and Methods

Connecting Statement (Chapter 3)
CHAPTER 5. MAPPING THE DOMAINS RESPONSIBLE FOR BIOLOGICAL
ACTIVITY IN THE LEISHMANIA GLYCOSOMAL PTS2 RECEPTOR PEX7.160

Abstract......................................................................................................................160

Introduction...............................................................................................................162

Materials and methods ..............................................................................................165
    Materials and reagents .........................................................................................165
    LmPEX7 modeling and sequence alignments....................................................165
    LmPEX7 constructs ...............................................................................................166
    Expression and purification of recombinant proteins ........................................167
    Leishmania transfection .......................................................................................168
    Protein binding assays .........................................................................................168
    Confocal microscopy analysis .............................................................................169
    Subcellular fractionation .....................................................................................169
    Western blot analysis .........................................................................................170

Results ........................................................................................................................171
    LmPEX7 modeling ...............................................................................................171
    Functional characterization of LmPEX7 ............................................................172
    Expression of LmPEX7-GFP fusion proteins .....................................................175
Immunofluorescence of LmPEX7-GFP fusion proteins.................................175
Subcellular fractionation..............................................................................179
Binding assays of GFP-tagged LmPEX7 mutants.........................................180

Discussion....................................................................................................185

Chapter 5 References ..................................................................................190

CHAPTER 6. SUMMARY AND CONCLUSIONS .............................................199

Chapter 6 References ..................................................................................206

Appendix 1. Permit from publishers...............................................................208
Abstract

The *Leishmania* peroxin 7 (LmPEX7 or LdPEX7) is the receptor that translocates PTS2 signal-containing proteins into the glycosome. This microbody is unique to and crucial for the survival of trypanosomatids which include *Leishmania* and *Trypanosoma*, the causative agents of leishmaniasis and African sleeping sickness, respectively. Proteins are imported into the glycosome via two pathways, PTS1 and PTS2, which involves the formation of a PTS-receptor complex in the cytosol, docking of the complex on a translocation apparatus on the glycosomal membrane, and subsequent release of the cargo protein into the lumen. However, the precise steps in glycosome protein trafficking are not well-defined and to understand the function of these organelles and prove their potential as chemotherapeutic targets, the mechanism of glycosome biogenesis needs to be fully elucidated. Not much is known about the mechanism of PTS2 import pathway in glycosomes as studies on PEX7 have been hampered by the difficulty in expressing a soluble recombinant form of this receptor. To dissect the PTS2 import pathway and to determine the role of PEX7 in *Leishmania*, this protein was cloned and characterized. LmPEX7 is a ~41 kDa protein containing six conserved WD40 motifs that displays limited sequence similarity to PEX7 homologues involved in the biogenesis of evolutionarily-related peroxisomes found in other eukaryotes. LmPEX7 interacts with PTS2 proteins, the PTS1 receptor LdPEX5, and the membrane-associated docking protein LdPEX14. These interactions, characterized through various biochemical techniques, were mediated by specific binding domains, formation of stable protein-protein complexes, and conformational changes. Based on subcellular localization studies, *Leishmania* PEX7 has a bimodal distribution and may function as a shuttling receptor which binds PTS2 proteins in the cytosol, targets to the glycosomal matrix to release PTS2, and recycles back into the cytosol. These studies contribute to the elucidation of glycosome function in trypanosomatids.
Abrégé

La péroxine *Leishmania* 7 (LmPEX7 ou LdPEX7) est un récepteur qui transloque les protéines qui contiennent le signal PTS2 dans le glycosome. Ce glycosome est unique et critique aux trypanosomes, tels que *Leishmania* et *Trypanosoma*, les agents causant la leishmaniose et la maladie Africaine du sommeil. Les protéines sont importées vers le glycosome par deux voies, PTS1 et PTS2, qui nécessitent la formation d’un complexe PTS dans le cytosol, l’amarrage du complexe sur un appareil de translocation sur la membrane du glycosome, et permet la libération de la charge protéique dans le lumen. Par contre, les étapes précises dans l’acheminement de protéines glycosomales ne sont pas bien définies et pour comprendre les fonctions de ces organelles et prouver qu’elles être des cibles chimiothérapeiques, les mécanismes impliqués dans la biogenèse doivent être très bien élucidés. Pour disséquer le mécanisme d’importation et pour déterminer le rôle de PEX7 chez *Leishmania*, cette protéine a été clonée à partir de l’ADN génomique de *L. major* et a été caractérisée. LmPEX7 est une protéine d’environ 41 kDa qui démontre une homologie limitée aux PEX7 impliqués dans la biogenèse des peroxysomes chez autres eucaryotes. LmPEX7 interagit avec les protéines PTS2, le récepteur PTS1 LdPEX5, ainsi que la protéine LdPEX14 qui est associée à la membrane du glycosome. Ces interactions, décrites en utilisant des techniques biochimiques variées, sont arbitrées par des domaines d’interactions situés sur LdPEX5 et LdPEX14, la formation de complexes protéiques stables, et associée à divers changements conformationnels. Des études de localisation subcellulaires ont permis de montrer que PEX7 a une distribution bimodale et fonctionne comme un récepteur navette qui lie les protéines PTS2 dans le cytosol, cible dans la matrice glycosomale pour relâcher le PTS2, et est retournée ensuite dans le cytosol. Ces études ont grandement contribués à l’élucidation des la fonction des glycosomes chez les trypanosomes.
**Contribution of authors**

**Chapter 3. Interaction of *Leishmania* PTS2 receptor peroxin 7 with the glycosomal protein import machinery**

Ana Victoria C. Pilar, Kleber P. Madrid, and Armando Jardim

AVCP performed the bulk of this study, prepared, and edited the manuscript. KPM assisted in preparing the LdPEX5 and LdPEX14 mutant constructs. AJ supervised the study and edited the manuscript.

**Chapter 4. Biochemical and biophysical characterization of the interaction dynamics between the *Leishmania* peroxin 7 and components of glycosome biogenesis**

Ana Victoria C. Pilar, Rona Strasser, and Armando Jardim

AVCP performed the bulk of this study, prepared, and edited the manuscript. RS assisted in performing the tryptic digestions. AJ supervised the study and edited the manuscript.

**Chapter 5. Mapping the domains responsible for biological activity in the *Leishmania* glycosomal PTS2 receptor peroxin 7**

Ana Victoria C. Pilar, Perunthottathu K. Umasankar, Line Dufresne, and Armando Jardim

AVCP performed the bulk of this study, prepared, and edited the manuscript. PKU cloned the *pXG-LmPEX7-GFP* construct. LD assisted in the cloning of LmPEX7 mutant constructs. AJ supervised the study and edited the manuscript.
Claims of originality

1. This is the first study to elucidate the mechanism of PTS2 import into the glycosomes in *Leishmania* by the cloning, expression, purification, and structural characterization of the recombinant PTS2 receptor LmPEX7. This is also the first report of the cloning, expression, and purification of the PTS2 protein fructose-1,6-bisphosphate aldolase in *L. donovani*.

2. Characterization of LmPEX7 by *in vivo* and *in vitro* assays showed that this receptor protein specifically binds the PTS2 signal of aldolase and interacts with the PTS1 receptor LdPEX5 and the docking protein LdPEX14.

3. The LmPEX7 binding domains on LdPEX5 and LdPEX14 were mapped using mutagenesis experiments and *in vitro* binding assays.

4. This is the first study to examine the nature of interactions between LmPEX7 and the various molecular components of glycosomal protein import using biochemical and biophysical techniques.

5. Mutagenesis and protein interaction assays showed that the N-terminal domain of LmPEX7 is important for binding LdPEX5 and LdPEX14.

6. Subcellular localization of *Leishmania* PEX7 demonstrated that it has a bimodal distribution in the cytosol and inside the glycosome.
Abbreviations used

**ALD** – aldolase

**APRT** – adenine phosphoribosyl transferase

**ATP** – adenosine triphosphate

**BSA** – bovine serum albumin

**DAPI** – 4',6-diamidino-2-phenylindole

**DME-L** – Dulbecco’s modified Eagle- *Leishmania* medium

**DNA** – deoxyribonucleic acid

**DTT** – dithiothreitol

**ELISA** – enzyme-linked immunosorbent assay

**FBS** – fetal bovine serum

**FITC** – fluorescein isothiocyanate

**GFP** – green fluorescent protein

**HGPRT** – hypoxanthine guanine phosphoribosyl transferase

**HRP** – horseradish peroxidase

**IMPDH** – inosine monophosphate dehydrogenase

**IPTG** – isopropyl-β-D-thiogalactopyranoside

**K_d** – dissociation constant

**Ld** or **Lm** – *Leishmania donovani* or *Leishmania major*

**LdPEX7** – native form of PEX7 in *L. donovani*

**LmPEX7** – recombinant form of PEX7 in *L. major*

**LPG** – lipophosphoglycan
MALDI-TOF – matrix-assisted laser desorption ionization-time of flight

NTA – nitrilotriacetic acid

ORF – open reading frame

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PEX – peroxin

PTS – peroxisomal targeting sequence

PVDF – polyvinylidene difluoride

RING – really interesting new gene

RNAi – ribonucleic acid interference

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS – Tris-buffered saline

TCA – trichloroacetic acid

TPR – tetratricopeptide motif

TX – Triton X-100

WD – tryptophan-aspartic acid

XPRT – xanthine phosphoribosyl transferase
Acknowledgements

“He who knows and knows that he knows is wise. Follow him.” - Kahlil Gibran

This thesis would not have been possible without the expert guidance of my supervisor Dr. Armando Jardim. My deepest gratitude for all the advice, knowledge, techniques, and skills you have imparted to me. I shall never forget them as I embark in my future endeavors.

To the co-authors of the manuscripts of this thesis – Pat, Rona, Line, and Umasankar – thank you for all your time, expertise, and friendship.

To my advisory committee members – Dr. Paula Ribeiro, Dr. Kris Chadee, and Dr. Herve Le Moual – thank you for all the helpful suggestions and comments.

This work was supported by grants awarded to Dr. Jardim from the Canadian Institute of Health Research, the Regroupements Stratégiques from Fonds de recherché sur la nature et les technologies Québec, and the Canada Foundation for Innovation.

To the Centre for Host-Parasite Interactions – thank you for providing travel funds so that I could present my research in various conferences.

To Jaime Sanchez and Michael Nyizstor – thank you for the technical assistance in confocal microscopy.

“A friend loves at all times.” – Proverbs 17:17

I shall never forget my former and present labmates most especially Rona Strasser, Celia Caballero-Franco, and Masaud Kakkar. Thank you for the invaluable support, camaraderie, and for sharing with me the highs and lows of graduate life. I will always
carry in my heart the best memories I have had with all of you. I love you all and wish you all the best.

“When all at once I saw a crowd, a host of golden daffodils…” – William Wordsworth

Life and work at the Institute of Parasitology would not have been fun and smooth without the help and support of all the faculty and staff. I thank them for their service and for creating a nurturing environment for the parasitology graduate students.

To my friends and fellow parasitology graduate students especially Peter Lee and Marie Claire Rioux – thank you for your warm hearts, kind words, and for helping me when I was the Parasitology student representative. Thank you for the shed tears of joy and sorrow for these bonded us together. Thank you to Maria Ait-Tyahity and Marie Claire for translating my abstract into French.

To Curtis Bosson – thank you for all the patience, support, roller-coaster rides, bushwhacking trips, and unforgettable (mis)adventures.

“When up a child in the way he should go, And when he is old he will not depart from it.”
– Proverbs 22:6

My mother and brothers have always been my source of strength and fountain of love. They inspired me to work hard and be the best that I could be. I dedicate this thesis to them.
List of tables

Table 1. Equilibrium dissociation constants for LmPEX7-LdPEX5, LmPEX7-Ldald 1-77, and LmPEX7-LdPEX14 interactions............................................................ 135

Table 2. Equilibrium dissociation constants for the LdPEX5-LdPEX14 and LdPEX5-LdXPRT interactions in the presence or absence of LmPEX7. ..............................135

Table 3. Half-life ($t_{1/2}$) of LmPEX7, LdPEX5, and LdPEX14 protein complexes in limited proteolytic digestion using trypsin.....................................................140
List of figures

Figure 1. Life cycle of *Leishmania sp.* ................................................................. 15
Figure 2. Diagram of the glycolytic pathway in trypanosomatids involving the glycosomes, mitochondria, and cytosol .......................................................... 37
Figure 3. Models of protein import in peroxisomes .................................................. 49
Figure 4. Expression and purification of LmPEX7 .................................................. 94
Figure 5. Functional characterization of LmPEX7 .................................................. 95
Figure 6. Interaction of LmPEX7 with other peroxin proteins .................................. 96
Figure 7. Mapping the LmPEX7 binding domain on LdPEX5 .................................. 100
Figure 8. The LmPEX7 binding domain on LdPEX14 ........................................ 101
Figure 9. Confocal immunofluorescence microscopy analysis of *Leishmania* PEX7 .... 102
Figure 10. Subcellular distribution of *Leishmania* PEX7 ..................................... 103
Figure 11. Orientation of LdPEX7 in the glycosome ........................................... 104
Figure 12. Model of PTS1 and PTS2 protein import into *Leishmania* glycosomes ...... 109
Figure 13. Structure analysis of LmPEX7 ............................................................. 131
Figure 14. Glycerol density gradient centrifugation of protein complexes ............. 132
Figure 15. Determination of equilibrium dissociation constants by ELISA-based protein interaction assays ................................................................. 136
Figure 16. Tryptic digestions of protein complexes .............................................. 142
Figure 17. Fluorescence measurements .................................................................. 144
Figure 18. Changes in surface hydrophobicity ..................................................... 147
Figure 19. Fluorescence quenching ....................................................................... 148
Figure 20. Predicted structure of LmPEX7 ............................................................ 173
Figure 21. Sequence alignments of N- and C- terminal regions of LmPEX7 .......... 174
Figure 22. Functional characterization of LmPEX7 .................................................. 176
Figure 23. Expression of GFP-tagged LmPEX7 in *L. donovani* promastigotes ........ 177
Figure 24. Confocal microscopy analysis of GFP-tagged LmPEX7 ......................... 178
Figure 25. Subcellular fractionation of GFP-tagged LmPEX7 .............................. 182
Figure 26. Protease protection assays ..................................................................... 183
Figure 27. Binding assays of GFP-tagged LmPEX7.....................................................184
Figure 28. Model of glycosomal protein import in *Leishmania* ........................................205
Chapter 1. General Introduction

The study of glycosome biology in the Family Trypanosomatidae is not only an exciting field for studying protein import in ancient and highly divergent organisms but also provides a platform for the development of drugs against diseases caused by the important parasites *Leishmania* and *Trypanosoma* [1, 2]. *Leishmania* parasites infect about 2 million people annually and the disease is endemic in 88 tropical and subtropical countries [3, 4]. There is currently no available vaccine against leishmaniasis and control efforts have been hampered by the parasite’s complex life cycle, lack of experimental animal models that correspond to clinical symptoms seen in humans, complications from HIV co-infection, and increased drug resistance [5-9]. The emergence of drug resistant *Leishmania* and lack of effective treatment strategies for leishmaniasis have led researchers to look into other therapeutic targets in this parasite among which glycosomes are considered prime candidates [1, 10].

Glycosomes are unique organelles found only in trypanosomatids and are morphologically and evolutionarily related to the peroxisomes in yeast and mammals [11, 12]. These microbodies house various metabolic activities such as glycolysis, purine salvage, β-oxidation of fatty acids, ether lipid, and isoprenoid synthesis [13]. Unlike peroxisomes, glycosomes in *Leishmania* and *Trypanosoma* do not have catalase but distinctively contain the first seven enzymes of glycolysis [14]. The importance of these organelles is exemplified in studies showing that the bloodstream form of *T. brucei* is completely dependent on glycolysis for ATP production and that interference with proper functioning of the glycosome is detrimental to the parasite [15]. However, the function of glycosomes in *Leishmania* is not yet fully understood since this organism utilizes substrates other than glucose for its energy supply [16].

Studies on glycosomes have primarily, but not exhaustively, focused on identifying the molecules involved in the import of glycosomal matrix proteins and targeting of glycosome membrane proteins [1]. Proteins involved in glycosome and peroxisome
biogenesis are called peroxins (PEX), about 30 of which have been identified in yeast and mammals but only 10-15 have been identified and characterized in trypanosomatids [10, 17, 18]. Since glycosomes do not contain DNA or protein translational machinery, glycosomal proteins are nuclear-encoded and synthesized in the cytosol on free ribosomes, and posttranslationally imported into the glycosomes without further proteolytic processing or modification [11, 13]. The import of glycosomal proteins involves two major classes of topogenic signals. The first signal is found at the C-terminus of the polypeptides called peroxisomal targeting sequence type 1 (PTS1) defined by the archetype tripeptide sequence Ser-Lys-Leu [19-21] while the second type, located at the N-terminus, is denoted by the nonapeptide sequence [(R/L)/(L/V/I)X5(Q/H)(L/A)] where X represents any amino acid residue [22-24]. PTS-containing proteins interact with the receptor PEX5 which recognizes PTS1 and PEX7 which binds PTS2 to form a ligand-receptor complex that docks on the translocation apparatus containing PEX14 on the glycosomal or peroxisomal membrane [18, 25-28]. The importance of the interaction between PEX5 and PEX7 as a further requirement for proper protein trafficking was demonstrated in RNAi experiments in T. brucei [33]. In L. donovani, PEX5 (LdPEX5) binds PTS1 via the conserved tetratricopeptide (TPR) repeats situated on the C-terminus of the receptor and the binding affinity of LdPEX5 for PTS1 is modulated by its interaction with L. donovani PEX14 (LdPEX14) which could serve as a trigger for the release and subsequent translocation of the PTS1 protein into the glycosomal matrix [29, 30]. Whether the same scenario occurs in the import of PTS2 proteins via the receptor PEX7 remains to be determined because not much is known about this arm of glycosomal protein import. Attempts to generate ldpex5 and ldpex14 null mutants in L. donovani have been unsuccessful suggesting that these proteins are essential for parasite viability [31, 32].

The precise mechanism of transport across the glycosomal membrane remains to be fully elucidated. Much of what is known about glycosome protein import has been based on peroxisome studies in yeast and mammals in which various models have been proposed to elucidate the mechanism of protein import [34, 35]. Whether the same translocation processes in peroxisomes are at play in glycosome biogenesis is unknown as various key
players involved in protein import have yet to be identified or characterized in trypanosomatids. It is important to note that the high divergence of trypanosomatids would warrant important differences in protein import mechanisms between glycosomes and peroxisomes [36] and such differences would be advantageous in designing drugs against leishmaniasis.
Thesis Objectives

The aims of this thesis are to elucidate the PTS2 import pathway in *L. donovani* through the following objectives:

- Cloning, expression, and purification of the *Leishmania* PTS2 receptor PEX7
- Characterization of the interactions between *Leishmania* PEX7 and the various components of the glycosomal machinery such as PTS2-containing proteins, LdPEX5, and LdPEX14, by using *in vitro* and *in vivo* protein-interaction methodologies, and mapping of the functional domains
- Determination of the subcellular localization and targeting of *Leishmania* PEX7 to glycosomes

Fulfillment of these objectives will give a better perspective on the relationship between the two protein import pathways, PTS1 and PTS2, and will ultimately contribute to the understanding of the function of glycosomes in *Leishmania*. 
Chapter 1 References


Chapter 2. Literature Review

*Leishmania* taxonomy

**Taxonomic classification**

The genus *Leishmania* (Ross 1903) belongs to Phylum Euglenozoa, Class Kinetoplastea, Order Typanosomatida, and Family Trypanosomatidae which also includes the genus *Trypanosoma*, and the mostly monogenetic parasites *Crithidia*, *Phytomonas*, and *Leptomonas* [1]. These protozoan hemoflagellates infect a wide range of hosts from invertebrates, plants to mammals [2]. There are about 30 species of *Leishmania* but only 23 are infective to humans. A related taxon, Family Bodonidae, differs from the trypanosomatids as they are biflagellated, free-living, ectocommensal or ectoparasitic on fish, and do not parasitise terrestrial vertebrates or plants [2]. Together, members of Class Kinetoplastea represent a major group in the field of parasitology.

*Leishmania* was first discovered by the Scottish physician named Sir William Boog Leishman in the 1900s. While serving with the British Army in India, he developed a stain called Leishman’s stain that detected an intracellular parasite in blood samples of patients with the disease kala-azar. Another researcher, Dr. Charles Donovan, independently discovered the causative agent of kala-azar while working in the Indian Medical Service. Drs. Leishman and Donovan published their accounts separately in 1903 and were both credited for discovering the protozoan parasite *Leishmania donovani* [3].
Characteristics of Kinetoplastids

Kinetoplastids are fascinating organisms because of their diversity, complexity, and unusual cell and molecular biology. The kinetoplastid species are differentiated on the basis of flagellar morphology, overall dimensions, and position of the kinetoplast which is a complex and condensed network of mitochondrial DNA near the basal body of the flagellum [2, 4, 5]. The kinetoplast contains thousands of concatenated circular DNA molecules made up of the maxicircles and minicircles. Maxicircles (20-38 kb in size) encode genes for ribosomal RNA and proteins involved in the electron transport chain and ATP synthesis. Minicircles (0.46-2.5 kb) contain genes for guide RNAs involved in RNA editing [6, 7].

Other biological peculiarities observed in kinetoplastids include a unique organelle called the glycosome which compartmentalizes glycolysis [8], extensive RNA editing [9], trans-splicing of a common 5’ leader sequence onto all mRNAs [10], antigenic variation [11], and discontinuous and polycistronic transcription [12]. The trypanosomatids contain novel cellular architecture such as a corset-like cytoskeleton made up of cortical microtubules [13], a multifunctional flagellar pocket used for endocytosis [14], excretion [15], and internalization of molecules such as glucose through various transporters [16], phagotrophy [17], a protective surface coating made up of glycoconjugates called glycocalyx that protects the parasites against the host immune response [11, 18], and a flagellum used not only for locomotion but also for host surface attachment in Leishmania [2].

Evolution of Kinetoplastids

A careful examination of kinetoplastid origin and evolution is essential for understanding the unique cell features and host associations in Leishmania. Analysis of divergence in the nuclear small subunit ribosomal RNA (SSU rRNA) show that the distant ancestors of
Kinetoplastids were probably free-living, bacterivorous, and flagellated jakobids [19], whereas phylogenies based on nuclear-encoded proteins purport that the facultative invertebrate parasites diplonemids are the closest relatives [20]. Within the kinetoplastid assemblage, the relationships of the various groups are not well-defined.

It is generally believed that the trypanosomatid lineage evolved from the eukaryotic lineage >600 million years ago [21]. Based on nuclear SSU rRNA sequences, massive evolutionary distances separate trypanosomatids from metazoans, and the appearance of trypanosomatids pre-dated the appearance of their current insect vectors as well as mammalian hosts [22]. Protein-based phylogenies based upon heat shock protein (HSP) markers and improved SSU rRNA analyses suggested that the trypanosomatids emerged late from the kinetoplastid line and are descendants of the bodonids [21, 23].

Parasitism may have evolved independently multiple times within the different kinetoplastid genera. The digenetic lifestyle (promastigote and amastigotes stages) emerged four times within the trypanosomatid lineage – in the African Trypanosoma sp., T. cruzi, Phytomonas sp., and in Leishmania sp. [21, 22]. The trypanosomatids probably originated from parasitic ancestors living inside blood-sucking insects that were accidentally transmitted into vertebrate hosts during a blood meal [21, 24]. Using mitochondrial rRNA gene sequences as a genetic clock, this event could have taken place ~300 million years ago which coincided with fossil evidence of ancestors of hemipteran insect hosts and animal reservoirs such as murid rodents, e.g. Old World rats and mice [25, 26].

Within trypanosomatids, phylogenetic studies contend that Trypanosoma is monophyletic and the earliest group to diverge from the trypanosomatid lineage [23]. The African trypanosome T. brucei and the American trypanosome T. cruzi represent two distinct clades within Trypanosoma owing to large genetic distances, differences in biochemical features and parasitic lifestyles [24, 27]. The other digenetic trypanosomatids, Phytomonas and Leishmania, are presumed to be monophyletic and emerged separately from the monogenetic taxa Crithidia and Herpetomonas [21].
The separation of *Leishmania* from *Trypanosoma* could have occurred around 100 million years ago before the breakup of the African and South American subcontinents and the emergence of the tsetse fly ancestors, the vectors for *T. brucei* and other African trypanosomes, as determined by fossil records [25, 27, 28]. Ancestors of New World species appeared in South America 46-36 million years ago and disseminated to Central and Southeast Asia [28]. The phylogeny of the *L. donovani* complex, the species causing visceral leishmaniasis in the Old World and New World, strongly correlates with geographical location. *Leishmania* species from Africa and India are more closely related than other *L. donovani*-complex groups in South America and Europe [28].

**Leishmania biology**

**Life cycle**

The digenetic life cycle of *Leishmania* consists of two main stages of growth and differentiation: promastigotes in a sandfly vector (Diptera: Phlebotominae) and amastigotes that reside inside phagocytic cells of a mammalian host (Fig. 1).

*Leishmania* are transmitted by the bite of a female sandfly during a bloodmeal. Inside the vertebrate host, the promastigotes are readily phagocytosed by macrophages where they differentiate into aflagellated, spherical amastigotes inside the phagolysosome. These forms divide and spread to other macrophages by lysing the initial host cell or by cycling from neutrophils to macrophages to reach target organs [29]. Amastigotes are taken up by a sandfly during a subsequent bloodmeal and the parasites then migrate to the gut wall of the vector and differentiate into non-infective procyclic promastigotes characterized by long, slender forms with a free flagellum measuring 6-8 μm in body length. These forms divide by longitudinal binary fission inside the peritrophic membrane that encases the bloodmeal, escape from the membrane into the midgut by secretion of a chitinase, and differentiate into the more slender nectomonad forms (15-20 μm) which begin forward
migration to the anterior midgut three days after the bloodmeal. Nectomonads attach to the midgut epithelium by inserting their flagella between the microvilli possibly through the glycolipid surface covering the parasites called lipophosphoglycan (LPG). From days 5-8, a second growth cycle is initiated after the nectomonads differentiate into short and broad leptomonads which are responsible for secreting the gel plug in the stomodeal valve of the sandfly. This plug accounts for the increased biting frequency of sandflies due to the regurgitation of blood during feeding and subsequent starvation of the vector. The leptomonads undergo metacyclogenesis to form highly motile, infective, and non-dividing metacyclic promastigotes to await transmission into another host [30-33].

The triggers for division and differentiation of the various forms of Leishmania are pH, temperature, hemin, exposure to sandfly saliva, and sugars when the sandfly is feeding on nectar (sugarmeal phase) [29, 33, 34].

Amastigotes differ from promastigotes based on decreased metabolic rate, increased proteinase and nuclease activities, expression of unique surface antigens, enhanced metabolic activity at acidic pH inside the phagolysosome, decreased expression of LPG, decreased expression of tubulins, accumulation of HSPs, increased expression of the surface protease gp63, and exclusive expression of the A2 family of proteins which consists of 45-100 kDa proteins [34-38]. The function of the A2 proteins in amastigotes is not yet fully understood but they are considered to be involved in virulence and pathogenicity.

Infective metacyclic promastigotes and amastigotes express a family of hydrophilic, acylated proteins called HASP and small, hydrophilic proteins found in the endoplasmic reticulum and mitochondrial outer membrane called SHERP. The genes encoding these proteins are found in the L. major cDNA16 locus and are considered to play a role in parasite virulence and amastigote survival [39, 40].
Promastigotes can differentiate into axenic amastigotes \textit{in vitro} by subjecting the cells to pH 5.5, 37 °C, and 5-7% CO₂ [41]. Axenic amastigotes have similar metabolic, morphological, and infective properties as macrophage-derived amastigotes [38].

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{Life cycle of \textit{Leishmania} sp. Modified from Bates (1994) [32].}
\end{figure}

**Medical importance of trypanosomatids**

\textit{Diseases caused by Leishmania sp.}

About 2 million people are infected by \textit{Leishmania} parasites annually and leishmaniasis is endemic in 88 countries [42, 43]. The global burden of leishmaniasis is important, causing a loss of 2.4 million disability adjusted life years (DALYs) (946,000 for males and 1,410,000 for females) [44].

\textit{Leishmania} causes four types of diseases:

- **Cutaneous leishmaniasis (CL)** – also known as oriental sore, is characterized by multiple skin lesions resulting in disfiguring scars. The lesions typically heal spontaneously
without treatment and patients who recover from CL remain immune to subsequent homologous infection [45]. The Old World species, *L. tropica* and *L. major*, and the New World species, *L. mexicana*, are causative agents of CL. About 1.0-1.5 million cases of CL are reported each year accounting for 50-75% of all *Leishmania* cases [46]. Ninety percent of these cases come from seven countries – Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria. An increasing number of cases of CL have been reported. In Brazil, 21,800 CL cases were reported in 1998 and this doubled to 40,000 in 2002 [44].

Visceral leishmaniasis (VL) – also known as kala-azar or black fever, is characterized by fever, abdominal pain, diarrhea, splenomegaly, and hepatomegaly. VL is caused by *L. donovani* (India and East Africa), *L. infantum* (Mediterranean countries), and *L. chagasi* (America). Kala-azar is often fatal if untreated and even after recovery, a chronic cutaneous form called post-kala-azar dermal leishmaniasis (PKDL) may develop and usually appears 6 months to 2 years after VL [47, 48]. This advanced form of disease is very infectious as the lesions harbor high doses of parasites [49]. For the year 2002, around 59,000 deaths due to VL (35,000 for males and 24,000 for females) were recorded [50]. VL is endemic in 62 countries with 200 million people at risk and around 500,000 new cases of VL are reported each year, 90% of which occur in only five countries: Bangladesh, Brazil, India, Nepal, and Sudan [43, 46, 51]. The northeastern Bihar state of India accounts for majority of these cases, e.g. 200,000 per year. VL caused two large epidemics in Bihar and a prolonged epidemic in Eastern Sudan in 1997 causing ~100,000 deaths [45]. Due to the serious pathology associated with VL, it has a higher priority than CL for control efforts [44].

Diffuse cutaneous leishmaniasis (DCL) – is less common than other forms of the disease and is a progressive and chronic form of CL that occurs in persons with a defective cellular immune response. The non-ulcerative lesions do not heal spontaneously and resemble leprosy. DCL is very difficult to treat and is often prone to relapse after treatment. The geographic distribution of DCL is restricted to Venezuela, Dominican Republic, Ethiopia, and Kenya [45].
Mucocutaneous leishmaniasis (MCL) – is also known as espundia and causes mutilating and disfiguring lesions on the face by destruction of the nasal mucous membranes, mouth, and throat. MCL can occur following resolution and cure of an initial CL caused by *L. braziliensis*. Ninety percent of MCL cases are found in only three countries: Bolivia, Brazil, and Peru. Other species, *L. donovani, L. major*, and *L. infantum*, have also been reported to cause MCL especially in patients infected with human immunodeficiency virus (HIV) [45, 46].

Leishmania-HIV co-infection

Visceral leishmaniasis is the fourth most common opportunistic disease in HIV-positive individuals [52]. At the end of 2003, approximately 38 million people in the world were infected with HIV. The geographical distribution of HIV-positive individuals overlaps with areas where *Leishmania* is endemic [53]. Thirty-four countries have reported cases of co-infection. In southern Europe, 25% to 70% of adult VL cases are related to HIV and 1.5% to 9% of AIDS cases suffer from newly acquired or reactivated VL [54, 55]. Most co-infections in South America are from Brazil while cases are increasing in Africa and Asia [45]. Co-infection is also observed in other immunosuppressed patients with hematologic neoplasia, organ transplant recipients, and patients on corticosteroid therapy [56]. The proposed mode of co-infection transmission is by sharing of needles and syringes among intravenous drug users [57]. Other people at risk especially in India and Africa are migrants, seasonal workers, refugees, sex workers, and truck drivers due to occupational exposure [53]. Despite the big impact of co-infections, data on optimal therapy for leishmaniasis in HIV patients are scarce. Treatment approaches are the same for both HIV-negative and co-infected patients. Antimonials are still the cost-effective drug of choice but the therapeutic responses are different: prolonged antimonial therapy is needed, there is increased frequency of relapses, and patients suffer more side effects [58]. If available, highly active antiretroviral therapy (HAART) is recommended to
reconstitute the host immune system. In India, Ambisome® is being used for treatment [54]. Oral miltefosine, although not yet tried on co-infections, is a promising cure [54].

Together, *Leishmania* and HIV elicit mutual aggravation of host immunosuppression [45]. HIV infection increases the risk of developing leishmaniasis by 100-1000 fold [59]. *Leishmania* promotes viral replication and decreases latent period of viral infection which ultimately progresses to AIDS, thus reducing the life expectancy of patients (13 to 19 months) [56]. Both HIV and *Leishmania* target macrophages and shift the immune response from Th1 to Th2, causing increased susceptibility to both diseases [55]. Aside from classic VL, the clinical features of HIV/*Leishmania* co-infection are: very low CD4+ T cell count, atypical manifestation of leishmaniasis involving the lungs and intestine, cutaneous dissemination of viscerotropic species causing DCL, lower frequency of splenomegaly in HIV patients with VL, and lower sensitivity to serological diagnosis due to lack of antibodies to *Leishmania* [45, 54].

*Diseases caused by Trypanosoma spp.*

Other medically important members of the Family Trypanosomatidae include *T. brucei gambiense*, *T. brucei rhodesiense*, and *T. cruzi*. The first two species are the causative agents of African sleeping sickness which is endemic in sub-Saharan countries and about 300,000 new cases are reported annually. African trypanosomes are transmitted by the tsetse fly and reside in the blood and tissue fluids of the mammalian host. Inside the host, trypanomastigotes appear as long, slender flagellates with the flagellum attached to the posterior end and runs along the entire length of the body [5]. The parasite is protected by a layer of variant surface glycoprotein (VSG) which undergoes frequent antigenic variation that enables the parasites to elude the host immune system [60]. Due to a lack of a functional mitochondrion, the bloodstream form is solely dependent on glycolysis for its energy production (substrate-level phosphorylation) [61]. Trypomastigotes differentiate into stumpy, non-dividing forms with no free flagellum and the mitochondria start to develop and function to a limited extent in cellular metabolism [62].
Trypomastigotes, when ingested by the vector, differentiate into morphologically similar procyclic trypomastigotes with well-developed mitochondria wherein metabolism is by oxidative phosphorylation [62]. The VSG coat is shed off and replaced by the surface protein procyclin in *T. brucei* [63]. The procyclic forms develop into epimastigotes and into the infective metacyclic forms upon migration to the salivary gland of the sandfly [5, 8].

*T. cruzi* causes Chagas disease which is endemic in the South American continent. About 3 to 5 million people are infected in Brazil alone [64]. The disease is characterized by acute meningoencephalitis and myocarditis and the infection becomes severe in immunocompromised persons [42]. Unlike African trypanosomes, *T. cruzi* is transmitted by reduviid bugs (*Triatoma* sp.) from which the metacyclic trypomastigotes are excreted in the feces and enter the host via the wound made by the blood-feeding bug [42].

Like leishmaniasis, there are few effective drugs currently available for treating African sleeping sickness and Chagas disease so that the development of new and improved therapeutic drugs is imperative [65, 66].

**Factors involved in the transmission and pathology of leishmaniasis**

*Parasite-vector interactions*

The ecological relationship between the host, vector, and the parasite is such that any changes in the behavior and environment of any of these will lead to changes in the epidemiology of the disease [67]. The distribution of leishmaniasis is limited by the geographical distribution of the sandfly vector. There are more than 300 species of sandflies in the world but only 32 species are proven vectors of *Leishmania*. The distribution of the disease is influenced by the susceptibility of the sandfly to cold climates, feeding behavior, and sandfly gut environment that would support the development of the parasite [44, 45, 68]. Specific vector-parasite pairings are required for
the transmission of Old World *Leishmania* species as these parasites have reduced survival inside the gut of an unnatural vector. In contrast, vectors from South America can transmit different New World *Leishmania* species [30].

Although *Leishmania* does not cause any pathology in the sandfly, evidences of modified feeding behavior and reduction in sandfly longevity have been observed [69, 70]. Alteration of sandfly feeding behavior is due to the gel plug secreted by the promastigotes in the anterior gut of the vector which effectively blocks the flow of blood through the stomodeal valve and causes blood regurgitation, thus preventing the sandfly from obtaining a bloodmeal and increasing biting persistency. Influence on sandfly behavior could serve to enhance transmission and increase parasite inoculum by causing the sandfly to try to probe and feed from several hosts [70, 71].

Sandfly saliva plays a role in establishing successful *Leishmania* infection in the mammalian host. Sandfly salivary components modulate the host immune response by decreasing macrophage activity, inhibiting interleukin-10 (IL-10) and tumor necrosis factor (TNF-α) secretion by antigen-presenting cells, causing inflammatory response that attracts monocytes and macrophages which are the ultimate hosts of the parasites, and driving the immune response to a Th2 type which renders the host more susceptible to infection (*reviewed in* [72]).

*Host-parasite interactions*

The outcome of leishmaniasis is influenced by the mutual interaction between host immune response and virulence of the parasite [67]. The parasite is able to adapt to man-made ecological changes by switching hosts from wild to domestic animals and peridomestic sandfly vectors [67]. Other sources of *Leishmania* transmission are via a zoonotic cycle – through an animal reservoir such as a dog or rodent; and anthroponotic – by inoculation from one person to another [53]. Environmental changes and individual risk factors that have brought about a concomitant increase in the incidence of
Leishmaniasis include: mass migrations from rural to urban areas due to civil unrest and occupational reasons, development projects like building of dams, roads, and irrigation which bring susceptible workers into contact with infected sandfly vectors, poor social and economic status, malnutrition leading to impairment of host immune system, use of immunosuppressive drugs, and HIV infection [44, 53]. The intrusion of people into sylvatic habitats by building farms and rural settlements and deforestation has also led to increased populations of the sandfly vector *Lutzomyia* and reservoir hosts in South America [45].

The major protective mechanism against *Leishmania* infection is by T-cell–mediated macrophage activation (CD4⁺ helper T cells, Th1 subset). After contact with antigen-presenting infected macrophages, T cells secrete interferon gamma (IFN-γ) and other cytokines to activate macrophages to kill the amastigotes by secreting nitric oxide (NO) and reactive oxygen intermediates through the NO synthase (iNOS) and NADPH oxidase systems. Susceptibility to infection is mediated by Th2 CD4⁺ type of response in which Th1 pro-inflammatory cytokines are suppressed and macrophages are deactivated [67, 73, 74].

*Leishmania* have evolved different mechanisms to survive inside the macrophage parasitophorous vacuole and evade the host immune system. *Leishmania* suppress IL-12 expression, IFN-γ production, and drive a Th2 type of immune response by inducing the expression of Th2 cytokines, IL-10 and tumor growth factor (TGF β), thus, preventing parasite killing by IFN-γ activated macrophages [67]. Once deposited inside the host, the parasites rely on passive uptake by macrophages or neutrophils by phagocytosis [75]. Two virulence factors, LPG and the surface protease gp63, promote parasite survival and permit differentiation to the amastigote stage inside the macrophage by inhibiting the maturation of the phagosome and degrading host lytic enzymes [76, 77]. The parasite is able to survive the respiratory burst because of the phosphorylated disaccharide units of LPG that scavenge oxygen intermediates [78]. Both LPG and gp63 protect the parasites from complement attack by removing the membrane attack complex (C5b-C9) from its membranes and converting C3b to C3bi to enhance uptake by phagocytosis [79].
Leishmania downgrades macrophage activity by inhibition of apoptosis, degradation of major histocompatibility complex (MHC) class II molecules, prevention of antigen presentation, inhibition of iNOS expression, and downregulation of macrophage co-stimulatory molecules and cytokines such as B7-1, IL-12, and the TNF-α receptor [75, 80]. The parasites increase macrophage longevity by delaying apoptosis possibly to allow parasites to reach target organs and promote dissemination [29, 75].

From a metabolic perspective, amastigotes subsist on amino acids, fatty acids, and lipids as major carbon and plasma membrane sources inside the phagolysosome. The parasites scavenge vitamins, purines, and metals from the host by using a battery of high-affinity membrane transporters, chelators, and proton-symporters (reviewed in [81, 82]).

Despite the increasing knowledge on the evasion strategies employed by Leishmania, very little is known about the precise timing, location, and sequence of events of these survival mechanisms. It is important to decipher how these successful parasites can persist inside the host for long periods of time yet allowing the host to survive without displaying overt clinical symptoms [75, 80].

**Diagnosis of leishmaniasis**

Early detection and treatment of leishmaniasis are needed to prevent host damage and death. Sensitive and specific diagnosis is imperative to differentiate leishmaniasis from other diseases of similar clinical manifestations, like leprosy or tuberculosis, and to distinguish acute from asymptomatic cases [48]. The gold standard is the demonstration of parasites in spleen aspirates (98% sensitivity). However, this painful procedure is complicated by hemorrhage, anemia, pregnancy (contraindication), need for trained medical workers, and time-consuming and expensive culturing of parasites [48].
Serological tools

Serological tools are based on the detection of humoral responses in the infected host. Antibodies against *Leishmania* are undetected 6 to 9 months after successful treatment. Serological techniques need to be more discerning in diagnosing leishmaniasis in seropositive infected individuals from non-infected but seropositive persons living in endemic areas [48].

**Indirect fluorescent antibody test (IFAT)** - This technique involves detection of circulating antibodies against *Leishmania* during the early stages of infection [51]. In this method, *L. donovani* promastigotes are fixed on slides and incubated with sera from infected patients to detect the presence of *Leishmania*-specific antibodies. The antibodies are subsequently probed with fluorescein-conjugated secondary antibodies and the slides are viewed under a fluorescent microscope. Positive samples show cytoplasmic or membrane fluorescence indicating antibodies bound to parasite antigens or epitopes [83]. IFAT is more sensitive and specific than soluble antigen ELISA and can be used to predict relapse. Drawbacks to IFAT are high cost, complicated procedures, and unsuitability in field conditions [84].

**Enzyme-linked immunosorbent assay (ELISA)** – Although a useful and valuable technique, the sensitivity and specificity of ELISA varies depending on the antigen used [85]. Of the recombinant antigens that have been evaluated, rK39, a kinesin-like 39-amino acid repeat found in *L. chagasi* amastigotes, has been the most promising and is proving to be highly sensitive and specific for VL cases because the antibodies are not present in CL and MCL [86]. The antibody titers correlate with the progression of VL and can be used to monitor therapy and relapse as the titers go down after successful treatment [86, 87].

**Direct agglutination test (DAT)** – In this technique, the host’s blood is diluted in microtiter plates and mixed with freeze-dried, stained promastigotes which are
agglutinated by the serum antibodies [48]. Important advantages of DAT are: high specificity and sensitivity, stability of the freeze-dried promastigotes, cost-effectiveness, and user-friendly procedures [44]. The development of a fast agglutination-screening test (FAST) circumvented the drawbacks of DAT by having shorter detection times of antibodies (<3 hours), using only one serum dilution, and having reproducible results. A DAT kit using the lyophilized antigen is now commercially available [43, 88].

**rK39 dipstick test** – This is a rapid test that detects antibodies to rK39 through an immunochromatographic test in the form of antigen-impregnated nitrocellulose paper strips (ICT) [85, 89]. It is sensitive and specific to VL in India, inexpensive, easy to use, and requires no equipment, making it ideal for field use [89]. An rK39 dipstick kit (InBios®, USA) approved by the Food and Drug Administration (FDA) is now commercially available [85].

**Antigen detection** – This is a more suitable and specific method than antibody detection especially in HIV/Leishmania co-infections where antibody levels are low [85]. The results of antigen detection in patient serum are affected by high levels of antibodies and immune complexes which may mask antigenic determinants and inhibit binding to free antigen. There is currently no antigen detection method available for CL and MCL. The latex agglutination test (KATEX) detects antigens in the urine of VL patients. It is very sensitive, specific for VL, works well with geographically-distinct samples, has good prognostic value, and is also valuable for diagnosing HIV co-infected patients (reviewed in [90]).

**Molecular diagnostic tools**

**Polymerase chain reaction (PCR)** – addresses the limitations imposed by serological methods. The advantages are: high sensitivity and specificity, early detection of parasite DNA or RNA, rapid use, usefulness in therapeutic follow-up, and detection of relapses especially in HIV co-infections [85, 91]. Unique parasite targets for PCR include the ITS
region, gp63 locus, rRNA genes, and kinetoplast DNA [92]. Recent advances in PCR-based assays include the use of SSU-rRNA sequences in HIV co-infected patients for monitoring relapse, development of species-specific primers to identify *Leishmania* at the species level [43], development of a PCR-ELISA technique for diagnosing MCL, and use of real-time PCR for quantifying parasite burden (*reviewed in* [85]). Despite the advances in PCR diagnosis, the problems still lie on high cost, unsuitability in field use, and the need for well-trained personnel [93, 94].

*Diagnosis of HIV/Leishmania co-infections*

Diagnosis of HIV/Leishmania co-infection is difficult. The gold standard is also demonstration of parasites in spleen aspirates (70-100% sensitivity). About 10 to 30% of VL cases are detected in HIV patients using tissue isolates from atypical locations such as skin, digestive, and respiratory tracts [95]. Serological methods are less useful in diagnosing co-infections due to low antibody responses in patients and higher positive serology in non-HIV immunosuppressed patients. Recent developments in antigen detection (KATEX) and PCR-based methods have contributed greatly in diagnosing HIV co-infections (*reviewed in* [56]).

*Summary statement*

To develop or improve diagnostic tools for leishmaniasis, various issues should be addressed such as lack of sensitivity and specificity, difficulty in field use, high cost, lack of commercial availability of antigens, lack of facilities, and need for prior technical skills. The development of diagnostic tools that would circumvent these drawbacks is currently being done by various researchers and the WHO-TDR (Special Program for Research and Training in Tropical Diseases) [93]. The future availability of better diagnostic tools would improve the control and management of leishmaniasis. This would
also lead to more successful treatment of the disease especially when it is detected during the early stages of infection.

**Treatment of leishmaniasis**

Drug therapy is still the major option for control of leishmaniasis since no effective vaccine is available. The following are drugs either currently being used to treat leishmaniasis or are in various stages of development and clinical trials:

**Pentavalent antimony (SbV)** – SbV (Sb$^{5+}$) is converted to its trivalent form (Sb$^{3+}$) inside the parasite which then induces apoptosis and increased efflux of thiols. This is the first line of treatment for leishmaniasis that has been in use for over six decades but a major drawback is the need for long parenteral courses. The emergence of large-scale resistance to this drug is exemplified in North Bihar, India where only 35% cure rate was observed [96]. Treatment failure of VL is correlated with geographical variations in SbV treatment regimens (low dose treatment, suboptimal prescribing, and production of low-quality drug), severity of disease, and variations in sensitivity of different *Leishmania* species [47]. Antimonials are not as effective in HIV co-infections because its mode of action also depends on a T cell response [56]. A generic sodium antimony gluconate (SAG; Albert David Ltd., Calcutta, India) which is less expensive and found to be as safe and effective as the more expensive brands such as sodium stibogluconate (Pentostam™; GlaxoSmithKline) and meglumine antimoniate (Glucantime, Aventis) is currently being marketed [47]. Combination chemotherapy is also being proposed to reduce resistance to SbV and studies are underway to determine safety and efficacy of drug combinations with amphotericin B [47].

**Pentamidine (isethionate or methansulphonate)** – This drug is slowly becoming obsolete as a second-line treatment for VL due to toxicity and resistance but it can still be useful in HIV/*Leishmania* co-infections [93]. In 2001, results of a randomized trial which
combined pentamidine and allopurinol had 91% efficacy compared to pentamidine alone (70%), proving that pentamidine could be used in combination therapy [47, 97].

**Amphotericin B** – This is a polyene antibiotic primarily used as an antifungal drug. Amphotericin B has higher affinity for 24-substituted ergosterol and forms aqueous pores in the membrane causing increased permeability and eventual killing of the cell [97]. *Leishmania* also has ergostane-based sterols in the membrane and this explains the high leishmanicidal activity of amphotericin B [47]. It is effective against antimony-resistant cases and MCL and it was recommended as the first line drug by the Indian National Expert Committee. Disadvantages of this drug are high cost, toxicity, need for hospitalization, and intravenous (IV) administration [47]. Resistance to amphotericin B is presently not a problem although relapses were seen after treatment of HIV co-infected patients [98]. Molecular studies correlate resistance to this drug to gene amplification which results in changes in the structure of membrane sterols [99].

**Lipid-associated amphotericin B** – The new formulations of lipid-associated amphotericin B are positive developments in leishmaniasis chemotherapy and are now considered as the leading drug in Europe and United States [47, 48]. The advantages of using this drug are improved tolerance due to reduced toxicity, extended plasma half-life, delivery using large doses over a short time, and efficacy in antimony-resistant cases [47]. AmBisome® (Liposomal amphotericin B (L-AmB); Gilead Sciences) was used to treat VL in India with high cure rates (89-97%) by using only one dose [100]. AmBisome® has been approved by the FDA and has the best safety profile among the other branded lipid-associated amphotericin. The major drawback is the high cost which is beyond the reach of poor patients [47]. Several approaches to reduce the cost were the use of cheaper lipid or nanoparticle formulations, use of one dose Ambisome®, and local manufacturing in India [101]. More recently, WHO announced a price reduction of Ambisome® for VL treatment in endemic areas [48].

**Miltefosine** – The most significant breakthrough in chemotherapy is the first oral treatment for VL. Miltefosine, an alkylphosphocholine drug which inhibits phospholipid
biosynthesis in *Leishmania*, was developed initially as an anticancer drug. Its efficacy in leishmaniasis was shown in the 1980s and further developed in the 1990s by Zentaris (formerly Astra Medica) and WHO/TDR with Phase III trials in India showing encouraging results (94% cured in 282 out of 299) [97]. It was registered in India in 2002 and approved for use. It is safe with low side effects and has a long half-life of 8 days. It still remains to be seen whether this drug is as effective in other endemic areas like Sudan and the Mediterranean [47]. However, miltefosine is mainly limited by its teratogenicity, so this excludes treatment of women of child-bearing age [102]. Combination therapy is recommended for treating HIV co-infected patients to prevent relapse [103]. More recently, resistance to miltefosine has been observed in *in vitro* studies so that its widespread use should be strictly controlled [48, 104].

**Paramomycin** – This aminoglycoside antibiotic which inhibits protein synthesis by binding to ribosomes is currently in its advanced stages of development. Completed phase II (India and Kenya) and III (India) trials proved the effectiveness and safety of this drug especially when used in combination therapy [105]. It was registered in India in 2006 [48]. Topical formulations of this drug (15% paramomycin) have been shown to be effective against CL [106].

**Sitamaquine (WR6026)** – This is an oral 8-aminoquinoline drug under development by GlaxoSmithKline and Walter Reed Army Institute for Research, USA. Little is known about its efficacy and small Phase I/II trials in Brazil, India, and Kenya had varying results. The mode of action is not clear but toxicity seems to be mild (*reviewed in* [107]).

**Imiquimod (imidazoquinoline)** – This is an ingredient of a commercially available topical cream for genital warts (Aladara™, 3M Pharmaceuticals). It is known to be an immunomodulator by inducing nitric oxide (NO) production in macrophages and was shown to have antileishmanial activity [108]. This drug was found to be effective in combination with meglumine antimony in resistant CL [47].
Antifungal azoles and other drugs – Interest in antifungal azoles has been due to the presence of similar ergosterols in *Leishmania* [97]. Although trials were limited, oral agents like ketoconazole were effective against *L. mexicana* while fluconazole healed CL (*L. major*) in 79% of patients in Saudi Arabia. Posaconazole showed activity against *L. amazonensis* in experimental models. More formal, well-designed trials are needed to determine the efficacy of these drugs (*reviewed in* [109]). Although still at late discovery phase, drugs such as biphosphonates (risedronate and pamidronate) for VL and CL [110] and a natural plant product, licochalcone A, from the Chinese licorice plant (*Glycyrrhiza glabra*) have shown antileishmanial activities [111].

**Summary statement**

Chemotherapy has been hampered by the development of drug resistance, high cost, toxicity, lack of information on the mode of action of most drugs, need for parenteral delivery, requirement of repeated doses, relapses especially in co-infections, differences in drug susceptibility of the *Leishmania* species, lack of funding for clinical and field trials, and lack of commercially available drugs (*reviewed in* [47]). There is still a need to develop more effective drugs and identify novel drug targets in *Leishmania* and *Trypanosoma*. Drug resistance is a major impediment to the future availability of drugs for the treatment of leishmaniasis. Understanding the mode of action of these drugs would provide insight into the mechanism of drug resistance in *Leishmania* parasites.

**Mechanism of drug resistance**

How *Leishmania* become resistant to some chemotherapeutic drugs is not well understood. Most studies that elucidate drug resistance were done using laboratory isolates and different *Leishmania* species so that the proposed resistance mechanisms need to be validated for field isolates [112]. Resistance is characterized by decreased drug
activity and response in the host. In the case of antimony, the pentavalent form has to cross the macrophage membrane to enter the phagolysosome and exert its effect on the amastigotes [112]. Both the pentavalent and the trivalent forms of antimony are effective against the two stages of *Leishmania* but it is not known how or which cell catalyzes the reduction of SbV to SbIII [112]. The mode of action of antimonials is not well-characterized but various studies have proposed that antimony interferes with glycolysis, induces apoptosis, and inhibits trypanothione reductase and glutathione reductase which would result in increased efflux of thiols and oxidative stress [113-115]. Resistance to antimonials could be due to the increased efflux or inhibition of uptake of the drug by decreased expression of membrane transporters such as aquaglyceroporins and increased expression of ABC transporters like the *Leishmania* homologs for the multidrug resistance-related proteins (MRP) which are known to confer drug resistance in cancer cells [112, 116]. MRP could also decrease antimony accumulation by coupling the drug with thiols for increased efflux similar to what was observed in yeast metal detoxification [117]. Similarly, the parasite either downregulates the expression of its own genes involved in thiol synthesis or modulates the genes in the host macrophage to decrease the reduction of SbV to SbIII [118, 119]. From these studies, development of drug resistance seems to be multifactorial and more studies are needed to understand the differences in resistance found in different *Leishmania* species and between laboratory and field isolates.

**Vaccine development**

Although there is still no available vaccine against *Leishmania*, considerable developments can be seen with advances in molecular techniques, genomics, proteomics, and completion of the *Leishmania* genome projects. The different vaccines against leishmaniasis fall into several categories:

**First generation or killed vaccines** – These vaccines consist of crude extracts from lysed or autoclaved parasites using a single species or mixture of *Leishmania* species and
administered with or without an adjuvant such as Bacillus Calmette-Guerin (BCG) [120]. First generation vaccines are more effective when used in combination therapy with antimonials [74].

Second generation vaccines – This category of vaccines include live-attenuated parasites, *Leishmania* genes cloned into recombinant bacteria or viruses for delivery, and *Leishmania* antigen-based vaccines *(reviewed in [73, 74])*). Attenuated parasites are not pathogenic and uptake into macrophages mimics that of their virulent counterparts. These parasites are produced either by long-term passage in liquid medium, irradiation, chemical mutagenesis, and mutagenesis by targeted deletion or knockouts of genes encoding for *Leishmania* virulence factors (cysteine proteases, dihydrofolate reductase, or gp63). However, these mutants induced very little protection in studies using murine models probably due to faster elimination by the host immune response *(reviewed in [73, 74])*.

“Leishmanisation” is an old practice in the Middle East and Eastern Europe where a live promastigote vaccine is used to produce resistance to CL infection and is the only vaccine proven to be effective in humans. Although protective, this was discontinued due to unacceptable and uncontrolled lesions in some patients. Scientists in Iran have now produced *L. major* stabilates (cells in genetically and viable condition) which should produce acceptable lesions [121]. Safer live vaccines are being formulated by including drug-sensitive *Leishmania* strains (with “suicidal drug cassettes”) [122].

Two vaccines based on purified *Leishmania* antigens have proven effective in dogs – FML saponin which was licensed for veterinary use and marketed as Leishmune® in Brazil is made up of the fucose-mannose ligand (FML) and saponin from the plant *Quillaja saponaria* as an adjuvant [123]; and LiESAP based on a 54 kDa protein secreted by *L. infantum* [124]. The following recombinant single or polyprotein vaccines were safe and effective in mice but only gave low to modest results in clinical trials using dogs and humans: 1) tandemly linked multi-subunit vaccine Leish-111f made up of thiol-specific antioxidant (TSA), *L. major* stress-inducible protein-1 (LmSTI-1), and
Leishmania elongation initiation factor (LeIF); 2) a vaccine consisting of H1 histone and hydrophilic acylated surface protein B1 (HASPB); 3) and the Leishmania homolog for activated C kinase receptors (LACK) administered together with IL-12 (reviewed in [73, 74]).

**Third generation vaccines** – DNA vaccination is a safer and less expensive alternative to other vaccines and elicits both humoral and cell-mediated immune responses in the host. However, DNA vaccines for leishmaniasis have not yet been developed for human use and more research is needed to prove its efficacy first in murine models as initial studies gave variable results possibly due to differences in plasmid use, dosage, Leishmania strains, and experimental procedures [73].

**Vaccines against sandfly saliva** – Compared to the other types of vaccines, sandfly saliva-based vaccines remain to be further explored. Although the mechanism of modulation of host immune response by sandfly saliva needs more investigation, it is worth knowing whether vaccines against salivary components could confer protection [73, 74]. Recent vaccines that have been shown to induce protection in mice were based on the saliva components Maxadilan, SP15 protein, and a mixture of Leishmania-killed vaccine and saliva extract [125-127].

**Summary statement**

Problems with vaccine development include the lack of animal models that mimic the clinical pathologies in visceral leishmaniasis, lack of cross-species protection and development of immunologic memory, and failure of various vaccines to confer protection to human hosts or dogs despite successful demonstration of efficacy in studies in other animals (reviewed in [73, 74]). For vaccines to be successful, these should be safe, affordable, induce long-term immunologic memory, confer cross-species protection, stable, and effective [73]. Vaccines for diseases such as leishmaniasis which mostly afflict poverty-stricken people are not very lucrative for most pharmaceutical companies.
and aid from developed countries is needed to alleviate this problem. WHO and the Bill and Melinda Gates Foundation have been instrumental in vaccine development against parasitic diseases by providing funding for research [73].

VL is a special case in vaccine design because of the unclear polarization of Th1/Th2 types of immune response in human hosts. Some vaccines that have been developed to drive a Th1 response with increased production of IFN-γ were not efficacious in animal trials whereas Th2-promoting vaccines show more promise in conferring protection suggesting that a Th1 response may not be required for immunity in VL cases (reviewed in [73]). Therefore, there is a need for studies to elucidate the Th1/Th2-mediated immune response of human hosts to VL. Vaccines should be developed that would induce the appropriate immune response.

Because of the role of vectors and animal reservoir in transmitting *Leishmania* parasites, these could be tapped as targets for chemotherapy or source of vaccines. Various strategies have been designed to stop parasite transmission by developing vaccines against sandfly saliva components and by controlling populations of animal reservoir.

**Vector and reservoir control**

The most common and widely used intervention for vector control (for endophilic sandflies) is insecticide spraying of houses. In Kabul and the Peruvian Andes, house spraying with pyrethroid lambdacyhalothrin reduced the risk of CL by 60% and 54%, respectively. Reduced indoor sandfly abundance was also observed in Venezuela where curtains were sprayed. These evaluations are important in determining the cost-effectiveness and sustainability of house spraying as cessation of spraying campaigns may lead to re-emergence of leishmaniasis (reviewed in [128]).

**Insecticide-treated bednets** – Bednets offer considerable protection from leishmaniasis as shown in studies in Bangladesh and Nepal and this is enhanced by treating bednets with
pyrethroids [128]. The use of treated bednets is a better alternative method to house spraying and more cost-effective [128]. Long-lasting nets are now available that would preclude re-treating and washing of nets [44].

**Pyrethroid-impregnated collars** – Control of zoonotic VL infections is done by elimination of infected dogs after the blood samples prove positive for *Leishmania*. This was shown to reduce VL incidence in dogs and children [129]. However, diagnosis and canine surveillance is labor-intensive and expensive [67]. Alternative methods of control include dipping of dogs in insecticide and topical application of insecticide [130]. Use of deltamethrin-treated collars resulted in a significant decrease in incidence of canine and human VL [129]. These collars are now commercially available but vaccination is probably the best strategy to control zoonotic leishmaniasis [48].

**Summary statement**

The development of chemotherapeutic drugs, vaccines against *Leishmania* parasites and sandfly salivary components, and reservoir control all contribute to the future success in managing transmission of parasites and treatment of the diseases. Other strategies such as identification of novel drug targets would greatly contribute to the development of more effective drugs against leishmaniasis. The unique biochemical and cellular features in *Leishmania* and other trypanosomatids such as the possession of a glycosome, RNA editing, and different transcriptional mechanisms are considered potential targets for chemotherapy [8-12].

**Glycosome biology**

The emergence of drug resistant *Leishmania* and lack of effective treatment strategies for leishmaniasis have led several researchers to look into other therapeutic targets in this
parasite. Glycosomes are membrane-bound microbody organelles unique to the Family Trypanosomatidae and have been considered as potential target for anti-

*Leishmania* drugs [65, 131, 132]. However, the mechanism of glycosome biogenesis has to be elucidated to demonstrate that this organelle can be useful as a drug target.

Glycosomes are morphologically and evolutionarily related to other microbodies like peroxisomes in yeast and mammals and glyoxysomes in plants [5, 133]. These organelles are very important in trypanosomatid biology because they function in various metabolic activities – glycolysis, purine salvage, β-oxidation of fatty acids, ether lipid biosynthesis, and isoprenoid synthesis [8, 134, 135]. The first seven enzymes of glycolysis are compartmentalized in the glycosomes so this organelle is essential for the survival of the bloodstream form of *T. brucei* which is dependent on glycolysis for ATP production [136]. However, the function of glycosomes in *Leishmania* is not yet fully understood because this organism utilizes substrates other than glucose for its energy supply [137]. An increase research interest in trypanosomatid glycosome has recently emerged which focuses on identifying and characterizing molecules involved in the import of glycosomal matrix proteins and glycosome assembly [132].

**General features of glycosomes**

Glycosomes were first described in the 1960s and were called glycosomes after the discovery of glycolytic enzymes in the matrix [8]. Much of the literature on glycosomes was based on studies in *T. brucei* due to the ease of manipulation of these parasites for biochemical and molecular studies. Structurally, glycosomes are spherical or elongated organelles with a diameter of about 0.2 to 0.3 μm [134]. A single phospholipid bilayer membrane surrounds an electron-dense matrix which does not contain any DNA [13]. The membrane is made up primarily of the phospholipids phosphatidyl choline and phosphatidyl ethanolamine [134]. Similar to other microbodies, glycosomes divide independently from the cell by binary fission [138]. The number of glycosomes in a trypanosomatid cell ranges from 200-300 in *Trypanosoma* bloodstream forms, 50-100 in
L. tropica and L. major promastigotes to as low as 10 glycosomes in L. mexicana amastigotes [139-141]. In T. brucei, glycosomes make up about 4% of the total cell volume and sediment at a density of about 1.23 g/ml in sucrose density gradients [134].

The evolutionary origin of glycosomes is still not clear although it has been proposed that these arose from a prokaryotic endosymbiont as similarly proposed for chloroplasts and mitochondria [138]. Determining the evolution of glycosomes is difficult because of the lack of DNA in these microbodies. In chloroplasts and mitochondria, much of the DNA from the endosymbiont was transferred to the nucleus and the organellar proteins synthesized in the cytosol are targeted to the organelles by topogenic signals [138]. The similarity of protein import and presence of multi-enzyme systems within the glycosome favor an endosymbiotic origin [138, 142]. Phylogenetic analysis of glycosomal enzymes such as triosephosphate isomerase and phosphoglycerate kinase showed that these enzymes are more related to the eukaryotic homologs, leading to the speculation that glycosomes probably originated from a eukaryotic endosymbiont [138]. The issue of whether microbodies such a glycosomes have mono- or polyphyletic origins has not yet been resolved [138].

**Enzymatic content of glycosomes**

Unlike peroxisomes, glycosomes of Trypanosoma and Leishmania do not contain catalase although this enzyme has been found in other trypanosomatids like Crithidia and Leptomonas [143]. Glycosomes share common enzymes with peroxisomes and glyoxysomes like those involved in β-oxidation of fatty acids and ether lipid biosynthesis. Glycosomes are distinct from other microbodies in that they house the first seven enzymes of glycolysis and two enzymes in glycerol metabolism [8, 139, 144]: hexokinase (HK), phosphoglucone isomerase (PIM), phosphofructokinase (PFK), fructose-1,6-bisphosphate aldolase (ALD), triosephosphate isomerase (TIM), glyceraldehyde-phosphate dehydrogenase (GAPDH), and phosphoglycerate kinase (PGK) (Fig. 2). The two enzymes found inside glycosomes that are involved in glycerol metabolism are
glycerol kinase (GK) and glycerol-3-phosphate dehydrogenase (GPDH). Glycosomes also have the last two enzymes in the pyrimidine biosynthetic pathway – orotate phosphoribosyltransferase and orotidine carboxylase [145]. Other enzymes include hypoxanthine guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase, inosine monophosphate dehydrogenase, guanine monophosphate reductase, and adenylate kinase which are involved in purine salvage [146-148]. The discovery of malate dehydrogenase (MDH) suggests that glycosomes undertake CO2 fixation [149]. Enzymes involved in ether lipid synthesis are phosphoenolpyruvate carboxylase (PEPCK), DHAP acetyltransferase, acyl COA reductase, and G-3-P:NADP oxidoreductase [133]. Analysis and purification of the enzymatic content of glycosomes (90% of which are glycolytic enzymes) show that glycosomal proteins make up about 8-9% of the total cell protein [135]. Other enzymes discovered in glycosomes such as sedoheptulose-1,7-bisphosphatase (SBPase) and glucose-6-phosphate dehydrogenase (G6PDH) which are part of the hexose monophosphate pathway bear resemblance to those found in algal chloroplasts or cyanobacteria and this is probably the result of horizontal gene transfer [150].

Figure 2. Diagram of the glycolytic pathway in trypanosomatids involving the glycosomes, mitochondria, and cytosol. Net ATP synthesis occurs in the cytosol where pyruvate kinase (PK) catalyzes the hydrolysis of phosphoenolpyruvate to pyruvate. NADH produced from glycolysis is oxidized by glycerol-3-phosphate oxidase found in the mitochondria. Figure taken from Parsons 2004 [151]. See text for details.
Advantages of glycosomes and glycolytic compartmentation

Glycolytic enzymes show stage-specific abundance differences [152]. Because bloodstream forms of *T. brucei* are solely dependent on glycolysis, the glycolytic enzyme levels are much higher than in the insect forms [153]. *T. brucei* bloodstream forms demonstrate one of the highest recorded flux rates of glycolysis among organisms [8]. However, net ATP synthesis does not occur in the glycosome but through substrate-level phosphorylation in the cytosol where 3-phosphoglycerate is converted to pyruvate producing only 2 ATPs and two pyruvate molecules for every glucose molecule [8, 61, 154]. The reduced form of nicotinamide adenosine dinucleotide (NADH) is regenerated through a glycerophosphate shuttle between the glycosomes and mitochondria. Glycerol-3-phosphate (G-3-P) produced in the glycosomes is reoxidized to dihydroxyacetone phosphate (DHAP) by the cyanide-insensitive mitochondrial terminal oxidase, sn-glycerol-3-phosphate oxidase (reviewed in [151, 155]) (Fig. 2).

It is not yet clear how the sequestration of glycolysis confers advantages to trypanosomes [156]. It was previously postulated that the compartmentation of glycolytic enzymes results in the high flux rates of glycolysis in trypanosomes which suggests that glycolytic substrates and products must cross the glycosomal membrane at high rates [8]. However, computer modeling showed that compartmentation does not affect the steady-state flux of glycolysis [156]. Nevertheless, glycosomes offer several advantages to trypanosomatids. Firstly, in contrast to other eukaryotes, trypanosomatid HK and PFK are not regulated and are not sensitive to allosteric inhibition during glycolysis. Limiting these enzymes inside the glycosomes prevents the danger of uncontrolled accumulation of intermediates and wasting of ATP during the first few irreversible reactions of glycolysis (called “turbo effect”). The total concentration of organic phosphates is also delimited by compartmentation. Therefore, glycosomes actually protect trypanosomatids by regulating HK and PFK and preventing metabolic interference as accumulated intermediates and glycosomal enzymes were found to be toxic to the parasites if these are not compartmentalized [156-159]. Secondly, compartmentation allows the parasite to recover from periods of starvation as it goes through its developmental cycle. The glycosome
prevents the depletion of ATP and insures the availability of phosphorylated intermediates to restart glycolysis and other biosynthetic pathways [132, 156]. Given these advantages, glycolysis could be the major reason for the evolution and maintenance of glycosomes in trypanosomatids [151].

Biogenesis of glycosomes

The glycosomal membrane is impermeable to molecules such as NADH, ATP, and intermediates of glycolysis so there could be specific translocators for these substrates [8, 160]. Although several transporters, mostly specific for lipid import, have been identified in peroxisomes, transporters in glycosomes have not yet been identified or characterized [151, 161]. The process of glycosome biogenesis is similar to that of peroxisomes in yeast and mammals and glyoxysomes in plants and different from the processes in mitochondria and chloroplasts [143]. Because glycosomes do not contain DNA or separate protein-synthesizing machinery, glycosome assembly would, therefore, need insertion of polypeptides into the glycosomal membrane and proteins have to be imported into the glycosomal matrix [8, 162]. Glycosomal proteins are nuclear-encoded and synthesized in the cytosol on free ribosomes and posttranslationally imported to the glycosomes. The transfer of polypeptides across the glycosomal membrane occurs without proteolytic processing and modification [8, 162]. This suggests that in contrast to chloroplasts and mitochondria, glycosomal proteins do not contain cleavable targeting signals. The translocation of glycosomal proteins after synthesis is very efficient, e.g. the polypeptides have a half-life of 1 to 3 min in the cytosol and 30 min to 1 h inside the glycosome [162, 163]. Little is known about how lipids and membrane proteins are recruited during glycosomal and peroxisomal assembly but it is postulated that the organelles are either constructed de novo or the membranes would come from the endoplasmic reticulum [132, 164].

Much of the current information on glycosome biogenesis is based on studies of peroxisome biogenesis. Peroxisome biogenesis has been a subject of intense study for the
past few years not only because of its metabolic functions but also due to its role in the human disease called peroxisome biogenesis disorder (PBD) [165]. Mutations and impairment in peroxisome activity in humans lead to genetic defects such as liver dysfunction, bone stippling (chondrodysplasia punctata), neurological impairment, and severe developmental defects that are generally fatal [166]. In other organisms like yeast, peroxisomes are not essential and mutants can easily be isolated to study peroxisome biogenesis [132]. In contrast, glycosomes are essential to trypanosomatids as interference in its proper functioning and knocking out genes involved in biogenesis such as PEX5 and PEX14 lead to a lethal phenotype or significant reduction in parasite virulence [136, 158, 159, 167, 168].

**PEX proteins**

The import of glycosomal and peroxisomal matrix proteins requires a set of proteins and cytosolic receptors while another set is needed for the insertion of proteins into the glycosomal and peroxisomal membrane [132]. Important and landmark data elucidating peroxisomal biogenesis and import processes have been obtained by studies on several species of yeast and Chinese hamster ovary cell lines deficient in peroxisome activity [169]. Relevant genes involved in peroxisomal biogenesis and import processes were identified by genetic complementation of these mutants, yeast two-hybrid systems, and functional assays [132]. Proteins that are involved in matrix protein import, membrane biogenesis, peroxisome proliferation, and organelle inheritance are categorized as peroxins or PEX proteins. A unified system of nomenclature was proposed for these proteins in 1996 [170]. At present, more than 30 peroxins have been identified in yeast and mammals but only 10-15 of these proteins have been found in trypanosomatids [66, 171]. Most of the *PEX* genes are evolutionarily conserved among eukaryotes [169]. In trypanosomatids, PEX genes were identified based on their homology with eukaryotic peroxisomal proteins. However, the trypanosomatid PEX proteins share low sequence identities with those of other eukaryotic species [151].
Glycosomal and peroxisomal import signals

Import of glycosomal and peroxisomal proteins involves classes of topogenic signals called peroxisomal targeting sequences (PTS).

**PTS1** - This signal, called peroxisomal targeting sequence type I (PTS I), is found at the C-terminus and is defined by a degenerate tripeptide sequence Ser-Lys-Leu (-SKL) and other variants consisting of small, charged, and hydrophobic residues. Most glycosomal and peroxisomal matrix proteins contain PTS1. There are differences in the acceptable degeneracy of the signals between trypanosomes and mammalian peroxisomes [172]. Addition of amino acids to the carboxyl terminus beyond the tripeptide, placement of the PTS1 signal to the amino terminal or at internal positions abolishes targeting of proteins to the peroxisomes [173, 174]. Based on the *L. major* genome, there are about 191 predicted PTS1 proteins, the majority of which bear the –SKL signal [175]. However, possession of a PTS1 does not mean that the protein targets to the glycosome or peroxisome [132].

**PTS2** - The second type of signal called PTS2 is found at the N-terminal end of the peptide and about 10 residues from the initiator methionine. The first enzyme possessing a PTS2 signal, 3-ketoacyl coenzyme A thiolase, was discovered in rat [176]. The PTS2 signal is defined by the nonapeptide sequence [(R/L)/(L/V/I)X5(Q/H)(L/A)] where X represents any amino acid residue [177]. The existence of the PTS2 import pathway in trypanosomes was demonstrated by the translocation of rat-3-ketoacyl-CoA thiolase and fructose-1,6-bisphosphate aldolase into glycosomes of *Trypanosoma* sp. [163, 172]. The crystal structure of aldolase from *T. brucei* and *L. mexicana* shows that the PTS2 signal in this homotetrameric enzyme forms a dimer which could be mediated by the hydrophobic residues in the signal sequence [178-180]. PTS2 proteins in other eukaryotes, like yeast thiolase and amine oxidase, could possibly have similar dimeric structures [180].
I-PTS - The third PTS type is an internal, hidden or bipartite targeting sequence found in peroxisomal proteins that do not have any identifiable PTS1 or PTS2 signals [181, 182]. The mechanism of import of these proteins still needs to be determined and distinct cytosolic receptors for these PTSs have not yet been identified [183]. It is hypothesized that internal PTSs facilitate import of oligomeric proteins by associating with other PTS-containing proteins probably through a “piggy-back” mechanism [184].

The reason why glycosomal and peroxisomal proteins contain two distinct PTS signals is unknown. Interestingly, some proteins such as fructose-1,6-bisphosphatase and mevalonate kinase in *Trypanosoma* contain dual PTS signals [175]. The dimerization of the PTS2 signals could mean that monomeric enzymes would settle for a PTS1 signal. However, glycerol kinase and glucose-6-phosphate isomerase are dimeric but contain a PTS1 instead. In the case of aldolase, the C-terminal tyrosine residue is important for catalysis so the PTS signal is logically found at the N-terminus. Surprisingly, other glycosomal enzymes with PTS1 in *Trypanosoma* have a PTS2 signal in *Leishmania* [175].

mPTS – The targeting and insertion of glycosomal and peroxisomal membrane proteins are independent from that of matrix proteins. Peroxisomal membrane proteins (PMPs) contain a signal sequence called mPTS consisting of basic amino acids associated with a transmembrane domain. Only three peroxins, PEX3, PEX16, and PEX19, contain mPTS and function in the import of membrane proteins during biogenesis [164].

**Glycosomal and peroxisomal PTS receptors**

PTS1 receptor

The *PEX5* gene encoding the PTS1 receptor was first identified in a *Pichia pastoris* *PEX5* mutant that was deficient only in the PTS1 pathway [185]. Homologs of this protein have been identified in several organisms like *S. cerevisiae, Homo sapiens,*
Yarrowia lipolytica, Candida boidinii, T. brucei, and L. donovani [167, 178, 183]. The N-terminal half of PEX5 contains up to seven pentapeptide repeats denoted by the motif WXXxF/Y [186] while the C-terminus contains up to seven tetratricopeptide (34 amino acid) repeats (TPR) [185] which function as the PTS1 binding domain [187]. The crystal structure of the TPR domain shows a helix-turn-helix motif with interacting anti-parallel \( \alpha \)-helices [188, 189]. Based on gel permeation chromatography, the human and Leishmania PEX5 exist as a homotetramer with the N-terminal region important for oligomerization [186, 190].

PEX5 interacts with numerous peroxins particularly the PTS2 receptor PEX7, the docking protein PEX14, PEX13, and the RING finger proteins PEX2, PEX10, and PEX12 [191-194] (Fig. 3). Both PEX13 and PEX14 are essential for the translocation of PTS proteins in peroxisomes with PEX14 representing the converging point of the PTS1 and PTS2 import pathways [195]. The pentapeptide motifs of human PEX5 are essential for interacting with PEX13 and PEX14 [196].

In humans, but not in yeast, there are two isoforms of PEX5, a short form (PEX5S) and a long form (PEX5L) which contains an additional 37 amino acid insert [193]. PEX5L interacts with the PTS2 receptor PEX7 and mediates binding to PEX14 on the peroxisomal membrane during PTS2 protein import. PEX5L functions similarly to the redundant proteins PEX18 and PEX21 which function as important accessory peroxins for PTS2 import in yeast and share similar PEX7 binding motifs [197]. This suggests that PEX5 must have acquired the function of both PEX18 and PEX21 in PTS2 import in mammals [197, 198]. PEX5 found in plants, L. donovani, and T. brucei is similar to the long isoform in mammals [167, 178, 199].

In mammals, PEX5 is found mainly in the cytosol with a small percentage (~5%) localized inside the peroxisomes. The targeting of PEX5 to the peroxisome is mediated by the first 110 amino acids at the N-terminus [200]. Studies on the subcellular localization of PEX5 gave rise to the model on receptor recycling where PEX5 bound to a cargo protein shuttles from the cytosol to the peroxisome [201] (Figure 3). In yeast and
mammals, PEX5 associates with the peroxisomal membrane and contains biophysical features similar to an integral membrane protein [185, 187, 202]. This gave rise to the proposed transient pore model of protein import in peroxisomes where PEX5 forms an oligomeric pore on the membrane to facilitate protein transport and shuttles back to the cytosol in an ATP-dependent manner and by a process of ubiquitination [203]. The export of this receptor from the peroxisome is mediated by the AAA ATPase proteins PEX1, PEX6, and PEX26 [194, 204] (Fig. 3).

Leishmania PEX5 (LdPEX5)

The *L. donovani* PEX5 (LdPEX5) is a 625 amino acid protein and has a molecular mass of 69.7 kDa. LdPEX5 exhibits structural features similar to PEX5 in higher eukaryotes e.g. seven conserved TPR motifs and three copies of the PEX5-specific pentapeptide sequence WXXX(F/Y). However, its overall amino acid sequence displayed limited similarity to other PEX5. Gel permeation chromatography experiments revealed that, similar to human PEX5, it behaves as a homotetramer in solution [167]. Unlike the mammalian and *Trypanosoma* PEX5, LdPEX5 binding to the *Leishmania* PEX14 (LdPEX14) does not involve the pentapeptide motifs and is instead mediated by a region spanning amino acids 290-323 [205, 206]. LdPEX5 undergoes conformational changes from a tetrameric to a dimeric form upon binding a PTS1 protein and the interaction of LdPEX5-PTS1 with LdPEX14 changes the binding affinity between LdPEX5 and the PTS1 cargo protein suggesting that LdPEX14 could be the trigger for the release of PTS1 into the glycosome [190]. In trypanosomatids, the subcellular localization of PEX5 is not yet clear but PEX5 could also be functioning as a cycling receptor [167].
**PTS2 receptor**

The PTS2 receptor PEX7 has been shown to bind specifically to the PTS2 sequence [207, 208]. PEX7 has seven WD40 motifs, a 44-60 amino acid sequence named for its conserved Trp-Asp dipeptide. Members of the WD40 family occur as β-propeller structures with up to seven propeller blades formed by the folding of the WD40 motifs and are known to have diverse functions in the cell such as transcription, signal transduction, and cell cycle regulation [207, 209, 210]. WD40 proteins also form oligomeric structures and interact with other proteins either through the repeat motifs or by the N- and C-terminal extensions commonly found in WD40 proteins [210]. Whether PEX7 adopts the same structure as WD40 proteins and uses the motifs for protein binding is not known. There is currently no three-dimensional structure for PEX7 and initial efforts to characterize it have been hampered by the difficulty in purifying this protein in soluble form [211]. The mammalian PEX7 was later successfully expressed and purified using a baculovirus system and the recombinant protein was demonstrated to occur as a monomer in solution although earlier studies have shown that PEX7 could also form dimers [211, 212].

Like PEX5, PEX7 is thought to function as a cycling receptor since it has been localized in the cytosol and inside the peroxisomes [213, 214]. Although the PTS2 import pathway in peroxisomes is not yet clearly defined, most researchers propose that it is similar to the PTS1 pathway as mutations or knockouts of genes involved in PTS1 import also resulted in impaired PTS2 targeting [212]. The main difference between the PTS1 and PTS2 pathways is the need for accessory or co-receptors for the targeting of the PEX7-PTS2 complex. PEX18/PEX21 in *S. cerevisiae*, PEX20 in *Yarrowia lipolytica*, and PEX5L in mammals are essential to PTS2 import and even share common structural features for binding to PEX7 [198]. Why PEX7 alone is not sufficient in importing PTS2 proteins into peroxisomes is not known. The proposed model for PTS2 import involves the binding of the cargo-loaded PEX7 to the accessory receptor(s) and formation of a docking complex made up of PEX14 and PEX13 on the peroxisomal membrane [212,
The whole length of PEX7 is required for targeting and protein-binding as 11-amino acid truncations either at the N- or C-terminal portions of PEX7 abrogated its biological activity [211, 216].

Little is known about the mechanism of PTS2 import in trypanosomatids. RNA interference of PEX5 or PEX7 in *T. brucei* affected the growth of this parasite and resulted in the mislocalization of PTS1 and PTS2 proteins showing that the interaction of these two receptors is essential for proper glycosome function [168]. Isolation and biochemical characterization of PEX7 in *Leishmania* has not yet been reported so that in order to fully elucidate glycosome protein import in this parasite, studies on PEX7 and identification of novel glycosomal proteins need to be done.

**Glycosomal and peroxisomal docking proteins**

PEX13 and PEX14 are two docking proteins that have been identified and characterized in yeast, mammals, and plants. The integral membrane protein PEX14 represents a point of convergence for both PTS1 and PTS2 pathways because it interacts with PEX5, PEX7, and PEX13 and mutants lacking PEX14 exhibit PTS1 and PTS2-deficient pathway phenotypes [195]. PEX14 was also found to interact with other peroxins such as PEX19 which is involved in peroxisome assembly [183]. The topology of PEX14 on or within the peroxisomal membrane is not clear but PEX14 could be forming large oligomers and associates with the peroxisomal membrane through a putative binding domain located at the N-terminus (residues 1-130) [217, 218]. The oligomerization domain is mediated by a coiled-coil motif at amino acid residues 147-278 in the mammalian PEX14 [217, 219].

The SH3 domain in the membrane protein PEX13 is important for interacting with PEX5 while the N-terminal domain mediates interaction with PEX7 [169, 220]. The interaction between PEX13 and PEX5 *in vitro* considerably diminished when PEX5 was pre-loaded with a PTS1 peptide. Since PEX13 associates with PEX5 and PEX14, it is possible that a PEX14-PEX5/PTS1 ternary complex promotes protein translocation through the
peroxisomal matrix and unloading of the PTS1 protein [221]. PEX13 could also be mediating the transfer of the PTS2 protein from PEX7 after the receptor-cargo complex docks on PEX14 [216].

\textit{Leishmania PEX14 (LdPEX14)}

PEX13 has not yet been isolated and characterized in \textit{Leishmania}. In the case of PEX14, the \textit{L. donovani LdPEX14} encodes a 464 amino acid protein with a predicted molecular mass of 48 kDa. Sequence alignments showed that LdPEX14 displays limited sequence similarity with PEX14 from other eukaryotes [222]. The conserved region from residues 23-63 at the N-terminus mediates binding to LdPEX5 [206]. Structural features of LdPEX14 include a predicted leucine zipper motif implicated in homodimerization and a putative transmembrane domain. In contrast to other PEX14 in other eukaryotes including \textit{Trypanosoma}, LdPEX14 behaves as a soluble protein that associates tightly with the glycosomal membrane as a peripheral membrane protein [222]. However, the topology of this protein on the glycosomal membrane has not yet been determined.

The affinity of LdPEX14 for LdPEX5 increases when the latter is loaded with a PTS1 cargo protein suggesting that LdPEX14 functions as a docking protein in glycosomes [206, 222] and similar results were obtained for \textit{T. brucei} PEX14 (TbPEX14) [136]. Unlike LdPEX14, the N-terminus of TbPEX14 protein binds specifically to the pentapeptide motifs WXXXF/Y of the \textit{T. brucei} PEX5 (TbPEX5). The trypanosomatid PEX14 is essential for proper glycosome functioning and parasite survival as demonstrated in RNAi experiments in \textit{T. brucei} [158].
Models of peroxisomal protein transport

The precise mechanism of glycosomal and peroxisomal transport still remains to be fully elucidated. Key molecules involved in peroxisomal protein transport participate either in forming stable complexes or transitory protein-protein interactions [132]. The first step in protein import of matrix proteins is the recognition of the PTS signals by cytosolic receptors PEX5 or PEX7 to form receptor-cargo complexes which move to the peroxisomal membrane and interact with the importomer complex made up of the docking proteins PEX13, PEX14, possibly PEX17, and the RING proteins PEX2, PEX10, and PEX12 which could be functioning as E3 ubiquitin ligases [223]. The receptor-cargo complexes then dissociate to translocate the cargo protein across the membrane and the receptors are either recycled back into the cytosol or degraded (reviewed in [203]). In the case of the PTS1 pathway, the release of the cargo protein into the matrix could be mediated by the interaction of PEX5 to PEX8 which causes the decrease in the PEX5-PTS1 affinity [224]. Receptor recycling occurs in an ATP-dependent manner through the AAA proteins PEX1 and PEX6 and by ubiquitination of PEX5 by the RING proteins [171, 225, 226].

Several models were proposed to illustrate the fate of the receptor-cargo complex after docking at the membrane (Fig. 3).

Simple shuttle model - the receptor delivers the matrix protein to the translocation apparatus, dissociates from the protein and recycles back to the cytosol (reviewed in [223, 227]).

Extended shuttle model - the whole receptor-cargo complex translocates across the membrane and releases the cargo into the matrix. The receptor is degraded inside the matrix or is exported out of the organelle. This model received a lot of support from various researchers after the subcellular localization of the PTS receptors was determined [171, 228].
Transient pore model – PEX5 was proposed to form a transient pore in the peroxisomal membrane by forming oligomeric subunits to translocate the PTS1 protein. The pore is disassembled in an ATP-dependent manner involving PEX8 and the AAA peroxins and PEX5 is recycled back to the cytosol by mono-ubiquitination or is targeted for degradation by proteosomes through poly-ubiquitination [203]. This is the most recent model for peroxisome protein import but more supporting evidence is needed to prove this model.

The identification of PEX6 and the RING proteins PEX2, PEX10, and PEX12 in T. brucei and Leishmania suggests that glycosomes also form similar docking or importomer complexes [229]. However, the involvement of these proteins in protein import still remains to be demonstrated.

Figure 3. Models of protein import in peroxisomes. 8, PEX8; Ub, ubiquitin. See text for details. Modified from Erdmann and Schliebs (2005) [203] and Rayapuram and Subramani (2006) [223].
Other cytosolic factors in glycosomal and peroxisomal transport

Other proteins or factors aside from peroxins may be involved in protein transport. However, the functions of these factors have yet to be determined. Although protein unfolding does not appear to be required for import into the glycosomes or peroxisomes, the heat shock protein (HSP) 70 was shown to interact with PEX5 and enhance PEX5-PTS1 binding [230]. Heat shock proteins are chaperones involved in protein folding in many cellular processes and may bind to proteins after synthesis to maintain the proteins in a loosely folded conformation to facilitate translocation across organellar membranes [231]. Antibodies directed against HSP70 inhibited protein import into peroxisomes suggesting that HSP70 might be playing multiple roles in protein import such as maintaining the targeted matrix protein in an import-competent state, stabilizing the region of the protein containing the PTS signal until it is bound by the appropriate PTS receptor, and increasing the accessibility of the PTS signal for receptor binding [232, 233]. Other heat shock proteins probably involved in peroxisomal protein import are HSP40, HSP60, and HSP90 (reviewed in [234]).

Potential drug targets in glycosomes

Reviews on glycosome biogenesis pointed out the various metabolic pathways in glycosomes as potential drug targets [65, 66]. However, there are currently no metabolic inhibitors developed specifically for these candidates. Because of the crucial functions of PEX5, PEX7, and PEX14 in trypanosomatids, these peroxins are also likely targets for drug design [132].
**Glycolysis**

Several of the glycolytic enzymes like aldolase, glycerol-3-phosphate dehydrogenase, and phosphoglycerate kinase are potential drug targets to inhibit glycolysis in trypanosomes [65, 66]. The PTS2 protein aldolase is a strategic enzyme for therapeutic agents because it is among the rate-limiting steps in glycolysis and because of its strategic position in the glycolytic pathway – at the junction of the hexose and the triose branches [180]. Computer modeling of the glycolytic flux in *T. brucei* also supports the view that inhibiting aldolase would greatly reduce the rate of glycolysis in the parasite [235].

**Ether lipid biosynthesis**

Ether lipids (alkoxyphospholipids) are characterized by the presence of ether linkage instead of the typical acyl linkage in other lipids [132]. Ether lipids are associated with the major surface molecules of the plasma membrane like LPG in *Leishmania* so by inhibiting LPG synthesis, this would render the parasite more susceptible to oxidative killing by macrophages.

**Purine salvage**

Trypanosomes do not synthesize purines *de novo* and, therefore, resort to salvaging purine metabolites from the host [147]. Unlike mammalian purine metabolic enzymes, the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT), adenine transferase, and xanthine phosphoribosyl transferase (XPRT) are exclusively glycosomal in trypanosomes and are, therefore, attractive drug targets [147, 148, 236].
Summary statement

To validate that glycosomes are essential to the survival of *Leishmania* and potential drug targets, the mechanisms required glycosome biogenesis need to be fully elucidated. By understanding the role of these microbodies in the parasite biology, inhibitors can be designed to effectively block glycosome function, leading to decreased parasite survival, virulence, or cell death.

The low sequence similarity of glycosomal proteins with their peroxisomal counterparts, unique glycosomal pathways, and high divergence in trypanosomatids suggest that there are differences between peroxisome and glycosome import mechanisms that may be exploited in drug design [151]. To this end, it is important to address how glycosome import occurs in trypanosomatids by studying the function and nature of interactions between the glycosomal proteins, identifying the components of the translocation complex, and determining the mechanism of receptor recycling.
Chapter 2 References


Connecting Statement (Chapter 3)

After the comprehensive review of literature, the following chapter is the first manuscript of the thesis in which the role of the PTS2 receptor protein PEX7 was investigated in *L. donovani*. The study involved the cloning, purification, functional characterization, and subcellular localization of the *Leishmania* PEX7. The format of this manuscript follows the guidelines set by the journal Molecular and Biochemical Parasitology, where it was published.
Chapter 3. Interaction of *Leishmania* PTS2 receptor peroxin 7 with the glycosomal protein import machinery

Ana Victoria C. Pilar, Kleber P. Madrid, and Armando Jardim

*Institute of Parasitology, McGill University, MacDonald Campus, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9*

*This manuscript was published in Molecular and Biochemical Parasitology 2008, Volume 158, Issue 1, pages 72-81.*

**Abstract**

*Leishmania* proteins containing a peroxisomal targeting signal sequence 2 (PTS2) are selectively trafficked to the glycosome by associating with the peroxin 7 receptor protein (PEX7). The *L. major* PEX7 (LmPEX7) encodes a ~41 kDa protein that exhibits limited sequence identity with PEX7 homologues from other eukaryotic organisms. Functional characterization of recombinant and native LmPEX7 revealed that this receptor bound the PTS2 protein fructose-1,6-bisphosphate aldolase. Moreover, LmPEX7 also formed a tight association with the *Leishmania* PEX5, the cytosolic PTS1 receptor, and PEX14, a glycosomal peripheral membrane protein required for protein import into the glycosome. Mapping studies revealed that the *Leishmania* PEX7 binds to a domain on LdPEX5 encompassing residues 111-148 and to a site on LdPEX14 spanning residues 120-148. Finally, subcellular localization studies revealed that *Leishmania* PEX7 has a dual distribution within the cytosolic compartment and glycosomal lumen.
Introduction

The kinetoplastid parasites Leishmania and Trypanosoma are the causative agents of a broad spectrum of human and veterinary diseases that include leishmaniasis, African sleeping sickness, Chagas disease, and “nagana” in cattle. Aside from their medical importance, these digenetic organisms have a number of idiosyncratic biochemical and metabolic features that distinguish them from other eukaryotes. The most notable of these include pan-editing of mitochondrial RNA transcripts [1], trans splicing of mRNA transcripts [2], organization of mitochondrial DNA into kinetoplastid structures and compartmentalization of glycolysis, purine salvage, and β-fatty acid oxidation pathways [3] within a microbody organelle known as a glycosome.

Glycosomes in Leishmania and Trypanosoma are morphologically and evolutionarily related to the peroxisomes in yeast, plants, and mammals. However, the hallmark enzyme catalase that is diagnostic of the peroxisomal microbody is absent from the glycosomes of these protozoan pathogens [3]. A number of genetic experiments have demonstrated that glycosomes are indispensable for the survival of the bloodstream form of Trypanosoma, as this stage of the parasite is largely dependent on glycolysis for energy production [3, 4]. In these parasites, genetic manipulations leading to the mis-targeting of glycolytic enzymes to the cytosol resulted in a conditional lethal phenotype when parasites were cultured in glucose-containing media [5]. A number of studies have suggested that glycosomes are critical for Leishmania survival, particularly for the amastigote stage where it must adapt to the hexose-restricted environment of the phagolysosome [6].

Glycosomal proteins are nuclear-encoded, translated on cytosolic polyribosomes, and posttranslationally imported into the glycosome [3]. Although little is known about the molecular processes involved in translocation of folded proteins across the glycosomal membrane, it has been demonstrated that proteins destined for the glycosomal matrix
generally contain either a peroxisomal targeting signal (PTS) 1 that consists of a C-terminal tripeptide with the archetype sequence Ser-Lys-Leu [7] or a more degenerate PTS2 signal which is typically located proximal to the N-terminus and has the consensus motif R/K-L/V/I-X5-Q/H-L/A [8, 9]. These two signals are recognized by the receptor proteins peroxin 5 (PEX5) [10-12] or peroxin 7 (PEX7) [13-15], respectively. More recently, mutagenesis studies in *T. brucei* have demonstrated that both PEX5 and PEX7 are required for proper protein import into the glycosome and parasite viability [16].

Faithful sorting and trafficking of nascent PTS1 polypeptides requires interaction of a PEX5-PTS1 binary complex with PEX14 on the glycosomal/peroxisomal membrane surface [11, 17-19] which is crucial for membrane translocation of PTS1 proteins [18, 20, 21]. A number of models have been advanced for PTS1 import in yeast and mammalian cells which include the extended shuttle model, where PEX5 is postulated to cycle between the cytosol and peroxisome lumen [11, 18, 22, 23], and the transient pore model, where loaded PEX5 is proposed to insert into the peroxisome membrane forming a pore through which PTS1 proteins are translocated [24]. In these models, PEX13, another membrane protein purported to work in concert with PEX5, PEX7, and PEX14, has been demonstrated to be an important component for protein import into peroxisomes [11, 18, 20, 25]. Whether PTS1 protein import into glycosomes proceeds via one of these two models is unclear as LdPEX5 has not yet been detected in the glycosomal lumen and extensive searches of the *Leishmania* and *Trypanosoma* databases have failed to detect a PEX13 homolog.

Unlike the PTS1 pathway, dissection of the PTS2 import mechanism has been less tractable. Genetic experiments have established that PEX7 is critical for targeting PTS2 proteins [9, 18]. However, difficulties in obtaining soluble full length recombinant PEX7 have limited the biochemical and biophysical analysis of the PEX7-PTS2 interaction [15, 26-28]. Using yeast two-hybrid approaches, PEX7 has been shown to interact with PEX5, PEX13, PEX14, PEX18 and PEX21, although several differences in the interaction patterns between mammalian and yeast PEX7 have been noted [15, 27, 29]. Association of mammalian and yeast PEX5 with a PTS2-laden PEX7 receptor has been
noted to be an important step for interaction with PEX14 and subsequent translocation of the PTS2 protein across the peroxisomal membrane [27, 30]. Mammalian cells express two PEX5 isoforms, however, only the long isoform (PEX5L) binds PEX7 [27]. In S. cerevisiae, PEX7 does not associate with PEX5 and PTS2 import appears to be accomplished by recruitment of PEX8 and PEX21 which are considered to be functionally similar to PEX5 [29, 31, 32].

In this study, we investigated the mechanism of PTS2 protein import into the glycosome by cloning the Leishmania PEX7 molecule and functionally characterizing the interaction of this receptor with PTS2 proteins and the Leishmania PEX5 and PEX14. In addition, immunocytochemical studies were performed to determine the subcellular localization of native PEX7.
Materials and Methods

Chemicals and Reagents

Restriction endonucleases, DNA-modifying enzymes, and chitin beads were purchased from New England Biolabs (Ipswich, MA). Secondary horseradish-peroxidase and dye-conjugated antibodies were procured from Sigma-Aldrich (St. Louis, MO) or Cedarlane Biologica (Ontario, Canada). Affigel-10 beads were purchased from Bio-Rad (Hercules, CA) and fungal protease K was procured from Invitrogen Life Technologies, Inc. (Grand Island, NY). All other reagents and chemicals used were of the highest quality commercially available.

Cell culture

Wild-type L. donovani (DI700) promastigotes were cultured in Dulbecco’s modified Eagle-Leishmania medium (DME-L) supplemented with 5 mg/L hemin and 100 μM xanthine (Sigma Aldrich).

Protein expression constructs

The LmPEX7 open reading frame (ORF) was amplified from L. major genomic DNA by PCR using the Pfx polymerase (Invitrogen) and the primer pair 5’-GGAATTCCATATGCGGCGGCGCTCCTCCAGAT-3’ (NdeI restriction site underlined) and 5’-CCCAAGCTTCCGAGGGAACGCGGTGG-3’ (HindIII restriction site underlined) with 30 cycles of 95 °C denaturation for 45 s, 56 °C annealing for 45 s, and 68 °C extension for 60 s. The PCR product was cloned into the NdeI-HindIII sites of the pET30b(+) vector (Novagen) to generate pET30b(+)-LmPEX7-His6.
The *L. donovani* fructose-1,6-bisphosphate aldolase ORF was amplified from genomic DNA using the *Pfx* polymerase and the primers 5′-GAATTCCATATGTCGCTGAGACGATC-3′ (*Ndel* restriction site underlined) and 5′-TATCTCAAGCTTATAGATGTTGCTTT-3′ (*HindIII* restriction site underlined) with 30 cycles of 94 °C denaturation for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 60 s, then cloned into the *Ndel-HindIII* sites of pET30b(+) to generate pET30b(+)-*LdALD-His6*. The pET30b(+)-*ldald-ΔPTS2-His6*, a mutant lacking the PTS2 (residues 1-9), was generated by PCR mutagenesis using the primers 5′-GATATACATATGTCGCCGGCGTGCAACCGC-3′ and 5′-GCGGTTGCACGCCGGCGACATATGATATC-3′, and pET30b(+)-*LdALD-His6* as the template.

The pTYB12-*ldpex5 111-181* construct was generated by PCR amplification using the primers 5′-CGAGCTATCCATATGTCGATGATGAATGCC-3′ (*Ndel* restriction site underlined) and 5′-AGCATCGAATTCCTGCTGGGGCTG-3′ (*EcoRI* restriction site underlined) and cloning this fragment into the *Ndel-EcoRI* sites of the pTYB12 expression vector (New England Biolabs). The pTYB12-*ldpex 1-148* expression construct was generated by digesting the pTYB12-*LdPEX5* plasmid (16) with *PstI* to eliminate a 1.3 kb fragment and re-ligating the vector. The pET30b(+)-*His6/S-ldpex14 1-148* expression vector was constructed by digesting pET30b(+)-*His6/S-LdPEX14* with *AatII* and *XhoI*, blunting the ends with T4 DNA polymerase, and re-ligating the vector. The sequence of all expression constructs was verified by automated DNA sequence analysis. Sequence alignments were performed using CLUSTALX [33].

**Protein expression**

*E. coli* ER2566 cells (New England Biolabs) transformed with pET30b(+)-*LmPEX7-His6*, pET30b(+)-*LdALD-His6*, pET30b(+)-*ldpex14 1-148* or pET30b(+)-*ΔPTS2-ldald-His6* were grown to ~0.7 OD<sub>600</sub> in LB containing 100 μg/ml kanamycin and protein expression was induced at 20 °C for 4 h with 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG).
Proteins were purified using Ni\textsuperscript{2+}-NTA resin as described by the vendor (Qiagen, Hilden, Germany). Wildtype His\textsubscript{6}/S-LdPEX14, LdPEX5 (LdPEX5WT), and various ldpex5 and ldpex14 fragments were prepared as previously described [10, 17, 19, 34]. ldpex5 111-181 and ldpex5 1-148 were expressed in \textit{E. coli} ER2566 as chitin-ldpex5 111-181 and chitin-ldpex5 1-148 fusion proteins and purified on a chitin column as outlined in the IMPACT-CN protocol (New England Biolabs). ldpex5 fragments were cleaved from the column-bound chitin fusion protein with 50 mM dithiothreitol (DTT) in 50 mM Tris-HCl pH 8.0 at 4 °C for 48 h. Recombinant proteins were concentrated and the buffer exchanged using a Biomax 5K NMWL centrifugal filter (Millipore, MA). The primary structure of the recombinant LmPEX7 was confirmed by MALDI-TOF/TOF tandem mass spectrometry sequence analysis at the UVic-Genome BC Proteomics Facility (Victoria, Canada). Recombinant LmPEX7 purified from inclusion bodies using Ni\textsuperscript{2+}-NTA resin under denaturing conditions was used to immunize guinea pigs (Cocalico Biologicals Inc., Reamstown, PA).

\textit{In vitro binding assays}

Affinity matrices were generated by coupling purified LmPEX7, LdALD, ldpex5NT, respectively, to Affigel-10 agarose beads (2-3 mg protein/ml of beads) in 50 mM sodium bicarbonate pH 8.0, 400 mM NaCl (SBS) for 16 h at 4 °C. Beads were washed with SBS and unreacted sites blocked with 100 mM ethanolamine in SBS buffer. For pull-down assays, 50 μl of packed affinity matrices were incubated with 15 μg of purified recombinant LdALD, LdPEX5, LdPEX14 or LmPEX7 in 50 μl 40 mM sodium phosphate pH 8.0, 500 mM NaCl (PS) for 30 min at 20 °C with occasional mixing. Beads were washed 3 x 1.0 ml PS-1% Triton X-100 (PST) and 3 x 1.0 ml PS to remove unbound proteins. For competition assays, LmPEX7 agarose beads were incubated with 2.5 μg of ldpex5NT and the unbound protein removed by washing beads with PBST. Charged beads were incubated with 1-, 10-, or 100-molar excess of ldpex5 1-148 for 20 min at 20 °C. Bound ldpex5NT was analyzed by Western blot using polyclonal rabbit anti-LdPEX5 antibodies. For native PEX7 pull-down assays, 1% Triton X-100/TBS
extract of *L. donovani* promastigotes (2 x 10^8 cells/ml) was mixed with 50 µl packed LdALD, ldald-ΔPTS2 or ldpex5NT agarose beads and incubated at 4 °C for 20 h with end-on-end mixing. Beads were washed 3 x 1.0 ml PST, 3 x 1.0 ml PS, and native *Leishmania* PEX7 was detected by Western blot analysis using anti-LmPEX7 antibodies.

Far-Western blot analysis was performed by resolving LdALD, ldald-ΔPTS2 and bovine serum albumin (BSA) (100 ng/lane) on a 10% SDS-PAGE and transferring proteins to a PVDF membrane. Membranes were blocked with 3% skim milk in 0.05% Tween-20 in PBS (PBST) and incubating with LmPEX7 (10 µg/ml in blocking buffer) for 2 h at 20 °C. Bound LmPEX7 was detected by Western blot analysis using anti-LmPEX7 antibodies.

*Western blot and confocal microscopy analysis*

Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad), blocked, and probed with rabbit anti-LdPEX5 (1:10,000), rabbit anti-LdPEX14 (1:10,000), rabbit anti-LdXPRT (1:2,000), rabbit anti-LdAPRT (1:1,000), guinea pig anti-LmPEX7 (1:5,000), or guinea pig anti-LdIMPDH (1:2,000) [10, 19, 35]. Primary antibodies were detected with goat anti-rabbit-horseradish peroxidase (HRP) conjugate (1:10,000) or donkey anti-guinea pig HRP (1:10,000) secondary antibodies, and blots developed with Western Lightning chemiluminescence reagent (PerkinElmer, MA). For confocal microscopy, *L. donovani* promastigotes were fixed with 4% paraformaldehyde in PBS, permeabilized and blocked with 0.25% Triton X-100, 2% fetal bovine serum (FBS) in 50 mM Tris-HCl pH 7.5 for 20 min. Promastigotes were probed with anti-LmPEX7 (1:100), anti-LdXPRT (1:100) or anti-LdAPRT (1:100) for 1 h at 20 °C [35]. Primary antibodies were detected with donkey anti-guinea pig FITC-conjugated or mouse anti-rabbit Cy3-conjugated secondary antibodies. Parasite nuclei and kinetoplastid DNA were counterstained with 4’-6-diamidino-2-phenylindole (DAPI) (50 µg/ml) for 10 min at 20 °C.
**Subcellular fractionation**

*L. donovani* promastigotes (2 x 10⁸ cells/ml) were resuspended in hypotonic buffer (5 mM HEPES pH 7.4, 2 mM EGTA, 2 mM DTT, protease inhibitor cocktail) 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 °C. Postnuclear lysate (5.0 ml) was 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°C in a Beckman SW41 rotor [36]. Gradients were fractionated from the top (500 µl/fraction) and proteins were precipitated with methanol-chloroform prior to Western blot analysis using anti-LmPEX7, anti-LdPEX5, anti-LdPEX14, anti-LdAPRT, or anti-LdXPRT antibodies.

A crude organelle fraction containing glycosomes was prepared by centrifugation of *L. donovani* postnuclear supernatants at 45,000 x g for 60 min at 4 °C. The pellet was resuspended in PBS and treated with 150 µg/ml protease K for 30 min at 0 °C in the presence or absence of 1% Triton X-100. Digests were terminated by trichloroacetic acid (TCA) precipitation and samples assayed for LdPEX7, LdPEX14, and LdIMPDH by Western blot analysis. The sensitivity of *Leishmania* PEX7 to protease K digestion was assessed by incubating recombinant soluble LmPEX7 (15 µg) or LmPEX7 bound to LdALD agarose beads with 20 µg/ml protease K at 0 °C for 30 min. The reactions were terminated by TCA precipitation and LmPEX7 was detected by Western blot analysis.

For digitonin permeabilization, *L. donovani* promastigotes were resuspended in 25 mM Tris-HCl pH 7.4, 250 mM sucrose, 150 mM NaCl, 1.0 mM EDTA (STES buffer) (1 x 10⁹ cells/ml) and 100 µl of cell suspension was mixed with 250 µl of STES containing digitonin at a final concentration of 3–360 µg/ml. Cell suspensions were incubated at 20 °C for 5 min, chilled on ice for 5 min, and the supernatant fraction was recovered by
spinning samples at 13,000 rpm for 2 min at 4°C. Proteins in the supernatant fractions were precipitated using TCA and analyzed by Western blot.
Results

Cloning and molecular characterization of LmPEX7

Mining the Leishmania genome databases with the Pichia pastoris PEX7, we identified an L. major gene encoding a putative PTS2 import receptor protein with a P value of $1.2 \times 10^{-30}$. L. major PEX7 (LmPEX7) encodes a 373 amino acid protein with a molecular mass of 40.8 kDa that shares 97% and 62% sequence identity with L. infantum and T. brucei PEX7, respectively. Pairwise alignments with the human, S. cerevisiae, or P. pastoris PEX7 showed that these proteins shared only ~25-44% sequence similarity with LmPEX7. However, a feature observed in LmPEX7, consistent with this protein functioning as a PTS2 receptor, is the five WD40 signature motifs that are conserved among all phylogenetically diverse PEX7 proteins. Another feature that distinguishes the kinetoplastid PEX7 from the yeast and mammalian PEX7 is a proline rich C-terminal domain.

Recombinant LmPEX7 containing a C-terminal hexahistidine tag expressed in E. coli ER2566 cells was recovered from both inclusion bodies and the soluble cytosolic fraction. SDS-PAGE analysis of LmPEX7 purified from the soluble fraction using Ni$^{2+}$-NTA chromatography showed a 41 kDa and a 60 kDa proteins that were identified by tandem MALDI-TOF/TOF sequencing as LmPEX7 and the E. coli chaperonin protein GroEL, respectively (Fig. 4A). LmPEX7 and GroEL were reproducibly co-purified even after numerous attempts to dissociate this interaction by washing with 1% Triton X-100, 1.0 M NaCl, 1.0 M MgCl$_2$, or 10 mM ATP. Expression of LmPEX7 variants containing an N-terminal hexahistidine tag did not eliminate induction or co-purification of GroEL. Despite the association with GroEL, LmPEX7 exhibited functional activity characteristic of PEX7 receptor proteins that was similar to the native Leishmania PEX7.

Western blot analysis of LmPEX7 purified from E. coli or native PEX7 in whole cell lysates of L. donovani promastigotes revealed that the guinea pig antisera raised against
LmPEX7 purified from inclusion bodies recognized a single 41 kDa immunoreactive protein (Fig. 4B, lane 2). Moreover, the immunoreactivity with native L. donovani PEX7 could be out-competed by pre-incubating anti-LmPEX7 antibodies with an excess of recombinant LmPEX7 (data not shown).

Characterization of recombinant LmPEX7 using L. donovani fructose-1,6-bisphosphate aldolase (LdALD) [37] revealed that LmPEX7 agarose beads bound LdALD but not the control protein bovine serum albumin (BSA) (Fig. 5A). SDS-PAGE analysis of the LmPEX7 agarose beads alone or beads exposed to BSA showed the presence of faint bands at ~41 kDa and 60 kDa that correspond to non-covalently attached LmPEX7 and GroEL (Fig. 5A). Similar experiments using lysozyme or BSA covalently coupled to agarose beads showed no binding of LdALD (data not shown), suggesting a specific interaction between this latter protein and LmPEX7. To verify that recombinant LmPEX7 selectively recognized and tightly bound PTS2 sequence, a Far-Western assay was performed using LdALD, ldald-ΔPTS2, and BSA as ligands. Membranes incubated with LmPEX7 showed that this receptor protein only associated with the full length aldolase containing PTS2 (Fig. 5B). These results demonstrate that LmPEX7 is structurally functional despite the presence of GroEL.

Incubation of L. donovani promastigote detergent extracts with LdALD (Fig. 5C, lane 1) or ldald-ΔPTS2 (Fig. 5C, lane 3) agarose beads revealed that native Leishmania PEX7 bound only to LdALD indicating that this receptor selectively recognized the PTS2 motif. As a control, aliquots of LdALD (Fig. 5C, lane 2) and ldald-ΔPTS2 (Fig. 5C, lane 4) agarose beads were also run on the Western blot to confirm that the immunoreactive band was derived from the Leishmania extracts.

**LmPEX7 protein-protein interactions**

Alignments of the L. major and L. donovani PEX7 indicated that the PTS2 receptors share 97% sequence identity. To examine the interaction of LmPEX7 with other
components of the glycosome biogenesis machinery, LmPEX7 agarose beads were mixed with recombinant ldpex5NT, an N-terminal fragment of LdPEX5 encompassing residues 1-391, recombinant LdPEX14, or a mixture of ldpex5NT and LdPEX14 (Fig. 6A). SDS-PAGE analysis of the pull down reactions revealed that LmPEX7 bound ldpex5NT and LdPEX14 (Fig. 6A, lanes 2 & 3). Incubation of LmPEX7 agarose beads with both ldpex5NT and LdPEX14 resulted in co-precipitation of ldpex5NT and LdPEX14 indicating that these proteins recognized unique binding sites on the *Leishmania* PEX7 (Fig. 6A, lane 4). Complementary experiments using S-protein beads that bind the S-peptide tag on recombinant LdPEX14 [10, 34] also confirmed the LdPEX14-LmPEX7 interaction (data not shown).

The capacity of native *Leishmania* PEX5, PEX7, and PEX14 to form a ternary complex was demonstrated by precipitation of LdPEX7 and LdPEX14 from *L. donovani* promastigote detergent extracts using ldpex5NT agarose beads (Fig. 6B, lane 1). Separation of pull-down reactions by SDS-PAGE and analysis of a dominant ~41 kDa band by tandem mass spectrometry analysis also revealed the presence of native aldolase in the pull down mixture. Western blot analysis revealed that the PTS2 protein hexokinase was also detected in the *L. donovani* promastigote pull down reactions (data not shown). In contrast, *Leishmania* extracts incubated with lysozyme agarose beads showed no detectable LdPEX7 or LdPEX14 (Fig. 6B, lane 2), confirming that the interaction of these latter proteins with ldpex5NT was specific. Moreover, Western blot analysis of the ldpex5NT agarose beads alone (Fig. 6B, lane 3) showed no immunoreactivity with anti-LmPEX7 and anti-LdPEX14 antisera.
Figure 4. Expression and purification of LmPEX7. (A) Recombinant LmPEX7-His$_6$ containing a C-terminal hexahistidine tag was purified by metal affinity chromatography. Co-purifying with LmPEX7 was a 60 kDa protein that remained tightly associated following stringent washes with buffers containing detergent and high salt concentrations. The identities of the ~41 and 60 kDa proteins were confirmed to be LmPEX7 and bacterial GroEL by tandem mass spectrometry analysis. (B) Polyclonal anti-LmPEX7 antibodies raised in guinea pigs specifically reacted with a ~41 kDa protein in the recombinant LmPEX7-His$_6$ preparations isolated from the cytosolic fraction of E. coli (lane 1) and native Leishmania PEX7 in whole cell lysates of L. donovani promastigotes (lane 2).
Figure 5. Functional characterization of LmPEX7. (A) LmPEX7 agarose beads containing covalently-coupled LmPEX7 were incubated with phosphate buffer saline (PBS) (lane 1), LdALD (lane 2), or bovine serum albumin (BSA) (lane 3) followed by washes with 1% Triton X-100, 0.5 M NaCl in PBS and bound proteins were analyzed by Coomassie blue-stained SDS-PAGE. (B) For the Far-Western analyses of the LmPEX7-LdALD interactions, 100 ng of LdALD (lane 1), ldald-PTS (lane 2) or BSA (lane 3) were resolved on a 10% SDS-PAGE and the proteins transferred to a PVDF membrane. Blocked membranes were incubated with 10 μg/ml recombinant LmPEX7 and then probed with anti-LmPEX7 antibodies and donkey anti-guinea pig HRP secondary antibody to detect bound LmPEX7. (C) The ability of native Leishmania PEX7 to bind PTS2 ligands was demonstrated by incubating LdALD or ldald-ΔPTS agarose beads with 1% Triton X-100 extracts of L. donovani promastigotes (+) or PBS alone (-). Leishmania PEX7 bound to the beads was assessed by Western blot analysis using anti-LmPEX7 antisera.
Figure 6. Interaction of LmPEX7 with other peroxin proteins. (A) LmPEX7 agarose beads were incubated with PBS (lane 1), 15 μg of ldpex5NT (lane 2), 15 μg LdPEX14 (lane 3), or a mixture of 15 μg each of ldpex5NT and LdPEX14 (lane 4). Beads were stringently washed with 1% Triton X-100, 0.5 M NaCl in PBS and proteins binding to the LmPEX7 agarose beads were analyzed by Coomassie blue-stained SDS-PAGE. (B) To determine if native *L. donovani* PEX7 and PEX14 bound LdPEX5, 1% Triton X-100 extracts of *L. donovani* promastigotes were incubated for 16 h at 4 °C with 50 μl of ldpex5NT agarose beads (lane 1) or lysozyme agarose beads (lane 2). As a control, ldpex5NT agarose beads were incubated for 16 h in PBS alone (lane 3). Proteins bound to the agarose beads were detected by Western blot analysis using a mixture of anti-LmPEX7 and anti-LdPEX14 antibodies.
Incubation of LmPEX7 agarose beads with a panel of LdPEX5/ldpex5 proteins revealed a robust association with full length LdPEX5, ldpex5NT, ldpex5 Δ1-111, and ldpex5 Δ269-291 (Fig. 7A & B, lanes 1, 2, 4, & 7). A reduced binding was observed with ldpex5 Δ181-314, a deletion mutant lacking residues 181-314 (Fig. 7B, lane 6). It is possible that the decreased binding activity with ldpex5 Δ181-314 may be due to a conformational change associated with the internal deletion. The ldpex5 111-181 fragment predicted to contain the LmPEX7 binding domain also bound to the LmPEX7 agarose beads (Fig. 7B, lane 9). In contrast, no binding was observed with ldpex5CT or ldpex5 203-391, fragments encompassing residues 303-625, and 203-391 respectively, or ldpex5 Δ1-202, a deletion mutant lacking the first 202 residues (Fig. 7B, lanes 3, 5 & 8). These results suggested that the LmPEX7 interaction domain was located at the N-terminus. Binding experiments with N-terminal fragment ldpex5 1-148 showed robust binding to LmPEX7 beads. Moreover, competition experiments revealed that a 100-fold molar excess of ldpex5 1-148 could displace the binding of ldpex5 NT to LmPEX7 (Fig. 7C). These results collectively indicate that LmPEX7 recognizes a binding site between residues 111-148 on LdPEX5.

Binding studies using the ldpex14 mutant proteins, ldpex14 Δ149-179 or ldpex14 Δ279-321 that lacked a putative hydrophobic region or a predicted leucine zipper motif, respectively [17], revealed that LmPEX7 bound wildtype LdPEX14 (Fig. 8, lane 1), ldpex14 Δ149-179 (Fig. 8, lane 2), ldpex14 Δ279-321 (Fig. 8, lane 3), ldpex14 1-322 (Fig. 8, lane 4), and ldpex14 1-148 (Fig. 8, lane 6). In addition to the full length LdPEX14, the pull down reactions also showed a number of additional immunoreactive bands that were due to LdPEX14 C-terminal proteolytic degradation products that also bound to LmPEX7 (Fig. 8, lane 1). No binding was observed with the N-terminal fragment, ldpex14 1-120 (Fig. 8, lane 5). Moreover, competition experiments using 100-fold molar excess ldpex14 1-120 showed that this fragment did not compete with ldpex14 1-148 or full-length LdPEX14 for LmPEX7 binding (data not shown). Together these
data indicate that LmPEX7 binds a 28 amino acid motif (residues 120-148) immediately upstream of the putative hydrophobic region on LdPEX14.

Subcellular distribution of the Leishmania PEX7

*L. donovani* promastigotes stained with anti-LmPEX7 antisera revealed a punctate pattern reminiscent of glycosomal organelles. Co-staining of parasites with anti-LmPEX7 and antibodies to the glycosomal enzyme xanthine phosphoribosyltransferase (LdXPRT) (Fig. 9, panels A & B) [35] showed co-localization of these proteins and suggested that the bulk of the *L. donovani* PEX7 was glycosome-associated (Fig. 9, panel C). Parasites co-stained with anti-LmPEX7 and antibodies to the cytosolic enzyme adenine phosphoribosyltransferase (LdAPRT) also suggested partial co-localization of PEX7 with LdAPRT (Fig. 9, panels D-F).

Biochemical fractionation of *L. donovani* promastigote postnuclear lysates on a linear sucrose density gradient revealed two populations of *L. donovani* PEX7, one co-sedimented with the glycosomal markers LdXPRT and LdPEX14 (Fig. 10A) at ~55% sucrose and the second was detected near the top of the gradient where the cytosolic proteins LdPEX5 and LdAPRT (data not shown) are normally found (Fig. 10A). However, since low levels of LdPEX14 and LdXPRT were observed in the cytosolic fractions, it was possible that the LdPEX7 present in the cytosolic fractions could be due to glycosome disruption during the cell lysis process. To ascertain if a population of LdPEX7 was present in the cytosolic compartment, *L. donovani* promastigotes were permeabilized with digitonin to differentially release proteins from the cytosol and subcellular organellar compartments. As shown in Fig. 10B, LdAPRT and LdPEX7 are released at similar digitonin concentrations indicating that they share a similar subcellular location. In contrast, significantly higher concentrations of detergent were required to promote the release of inosine monophosphate dehydrogenase (LdIMPDH) from the glycosome [38]. Together these data suggest that *Leishmania* PEX7 has a dual distribution between the cytosolic compartment and the glycosomal matrix.
To determine if the population of LdPEX7 molecules purifying with the glycosomes were associated on the cytosolic face of the glycosomal membrane or present in the glycosome matrix, a protease protection assay was performed. Treatment of an *L. donovani* promastigote postnuclear fraction or purified glycosomes with 150 μg/ml protease K showed that LdPEX7 and LdIMPDH exhibited comparable resistance to proteolysis indicating that a portion of LdPEX7 present in the glycosomal lumen was protected from degradation (Fig. 11A). However, disruption of the glycosomal membrane with Triton X-100 resulted in complete degradation of LdPEX7 and LdIMPDH demonstrating that both proteins were sensitive to protease K (Fig. 11A). In contrast, LdPEX14 which is anchored to the glycosome surface was readily degraded by protease K in the presence or absence of Triton X-100 (Fig. 11A).

In the mammalian system, a recent report suggested that the preloading of PEX7 with a PTS2 ligand increased resistance of this receptor to proteolytic digestion [39]. Treatment of recombinant LmPEX7 alone (Fig. 11B) or LmPEX7 bound to the LdALD beads (Fig. 11C), showed that in both cases, LmPEX7 was rapidly degraded by protease K. This argues against the possibility that the observed resistance of native *Leishmania* PEX7 to proteolytic degradation was due to the formation of LdPEX7-PTS2 complex at the glycosome surface.
Figure 7. Mapping the LmPEX7 binding domain on LdPEX5. To localize the LmPEX7 binding domain, 15 μg of various mutants or fragments of LdPEX5 represented in the schematic diagram (A) were incubated with 50 μl of packed LmPEX7 agarose beads. Agarose beads were stringently washed and LdPEX5/ldpex5 proteins binding to LmPEX7 beads were detected by Western blot analysis using anti-LdPEX5 antibodies (B). White boxes correspond to the pentapeptide repeats (WXXXF/Y) and cross-hatched boxes denote the tetratricopeptide repeats. Lane numbers in panel B correspond to the protein constructs shown in panel A. (C) For competition assays, LmPEX7 agarose beads were pre-charged with 2.5 μg of ldpex5 NT, unbound proteins were removed, and the beads were then incubated with 1-, 10-, or 100- molar excess of the fragment ldpex5 1-148. Bound ldpex5 NT was detected by Western blot analysis using anti-LdPEX5 antibodies.
**Figure 8. The LmPEX7 binding domain on LdPEX14.** A series of LdPEX14 fragments or deletion mutants represented in the schematic diagram (A) were constructed based on bioinformatic analysis of the LdPEX14 primary structure. The LdPEX5 binding domain on LdPEX14 is represented by the *white box* and the *cross-hatched box* denotes a predicted hydrophobic patch within residues 155-179. (B) LmPEX7 agarose beads were incubated with 15 μg of wildtype LdPEX14 (lane 1), ldpex14 Δ149-179 (lane 2), ldpex14 Δ279-321 (lane 3), ldpex14 1-322 (lane 4), ldpex14 1-120 (lane 5), ldpex14 1-48 (lane 6) and after stringent washes, the bound LdPEX14/ldpex14 was detected by Western blot analysis.
Figure 9. Confocal immunofluorescence microscopy analysis of *Leishmania* PEX7. (A) *L. donovani* promastigotes were fixed with paraformaldehyde, permeabilized with 0.25% Triton X-100 in PBS, and incubated with guinea pig anti-LmPEX7 (*panels A & D*), rabbit anti-LdXPRT (*panel B*), or rabbit anti-LdAPRT (*panel E*) primary antibodies. Primary antibodies were visualized by staining with goat anti-guinea pig FITC-conjugated or mouse anti-rabbit Cy3-conjugated secondary antibodies. Panels C and F are merged confocal microscope images of parasites doubly stained with anti-LmPEX7 and anti-LdXPRT or anti-LmPEX7 and anti-LdAPRT, respectively. Nuclear and kinetoplastid DNA were stained with DAPI (blue stain). Images were collected at 1000X magnification.
Figure 10. Subcellular distribution of *Leishmania* PEX7: (A) *L. donovani* promastigote postnuclear lysates were fractionated on a linear 20–60% sucrose gradient. Fractions were assayed for the presence of LdPEX7, LdPEX5, LdPEX14, and LdXPRT by Western Blot analysis. LdXPRT is a lumenal glycosomal protein; LdPEX14 is a peripheral membrane protein anchored on the cytosolic face of the glycosome; and LdPEX5 is a cytosolic receptor. (B) The subcellular distribution of LdPEX7 was also examined by differential digitonin permeabilization of the plasma membrane. *L. donovani* promastigotes (1 x 10^8) were incubated with increasing concentrations of digitonin (3-360 μg/ml) and the supernatant fractions were precipitated with trichloroacetic acid and the release of LdPEX7, LdAPRT, and LdIMPDH was assayed by Western blot analysis.
Figure 11. Orientation of LdPEX7 in the glycosome. (A) To determine if the portion of LdPEX7 associated with glycosomes was situated on the glycosomal surface or within the lumen, postnuclear lysates of *L. donovani* promastigotes were treated with protease K (150 μg/ml) for 30 min at 0 °C in the presence or absence of Triton X-100. Digests were assayed for LdPEX14, a glycosomal membrane surface protein, LdIMPDH, a glycosomal matrix protein, and LdPEX7. The capacity of PTS2 ligand to alter the *Leishmania* PEX7 susceptibility to proteolytic degradation was examined by treating LmPEX7 (15 μg) in solution (B) or bound to LdALD agarose beads (C) with protease K (20 μg/ml) for 30 min at 0 °C. Undigested *Leishmania* PEX7 was detected by Western blot analysis.
Discussion

Here we present the functional characterization of the native and recombinant *Leishmania* PEX7 PTS2 receptor. Previous attempts to biochemically examine the interaction of PEX7 with a PTS2 ligand using a molecularly defined *in vitro* system have been hampered by difficulties in the production of soluble recombinant PEX7 in bacteria [15, 26-28]. However, Mukai *et al.* [39] recently reported the expression of a soluble form of recombinant mammalian PEX7 in baculovirus. In this study, full length recombinant *Leishmania* PEX7 was expressed in *E. coli*. However, LmPEX7 was found to co-purify with the chaperonin GroEL. Attempts to dissociate GroEL from LmPEX7 using high concentrations of Mg$^{2+}$ or ATP were not successful. In eukaryotic cells, the cytosolic chaperonin TRiC/CCT, the counterpart to GroEL, has been implicated in the folding of WD40 proteins and in particular, PEX7 in yeast [40]. Although GroEL has been suggested to lack binding and refolding activity with WD40 proteins [41], in this study, GroEL forms a tight association with conformationally functional recombinant LmPEX7. Like native *Leishmania* PEX7, the recombinant receptor selectively bound PTS2 ligands and associated with LdPEX5 and LdPEX14 despite the presence of GroEL. In *Leishmania*, the folding of nascent PEX7 may likely be mediated by a TRiC/CCT homologue [40]. Sequence alignments of the *Leishmania* and *S. cerevisiae* t-complex protein 1, a subunit of the TRiC/CCT complex, indicated that they share ~60% sequence identity (data not shown).

An important step in the trafficking of PTS2 proteins to the peroxisome or glycosome is the association of PTS2-laden PEX7 receptor with the membrane-anchored protein PEX14 [20, 32, 42]. *Leishmania* PEX7 interacts directly with LdPEX14 in the presence or absence of a PTS2 ligand. Moreover, *Leishmania* PEX7 also binds PEX5 independently of PEX14. This contrasts with observations in other systems where import of PTS2 proteins is dependent on the PEX5L, PEX5, or PEX18-PEX21, respectively, for the docking of PEX7 with PEX14 [27, 29, 30]. These proteins share a 37 amino acid structural motif which constitutes the PEX7 binding site initially identified in the
mammalian PEX5L [27, 43]. A recent report has suggested that based on sequence alignments, the *Leishmania* PEX7 likely recognized a motif on LdPEX5 situated between residues 193-249 [16], however, our domain mapping studies indicated that *Leishmania* PEX7 interacts primarily with a structural element on LdPEX5 located between residues 111-148, a region which shares no sequence similarity with the 37 amino acid insert in the mammalian PEX5L. Although recombinant *Leishmania* PEX5 and PEX7 form a stable complex *in vitro* in the absence of PTS1 and PTS2, it is unclear if analogous binary interactions occur *in vivo* since pull-down experiments using ldpex5NT beads precipitated a complex containing LdPEX14, LdPEX7, as well as the PTS2 protein aldolase. Similarly, pull-down experiments using aldolase agarose beads precipitated a complex that contained LdPEX5, LdPEX7, and LdPEX14 from detergent extracts of *L. donovani* promastigotes (data not shown).

The *Leishmania* PEX7 receptor, unlike the mammalian and *Arabidopsis* proteins [27, 42, 44], binds directly to LdPEX14 within residues 120-148, a region downstream of the LdPEX5 binding motif (residues 23-63) [17]. Despite the finding that the *Leishmania* PEX7-PTS2 complex can dock directly to LdPEX14 on the glycosomal surface, this interaction alone is not likely to be sufficient for translocation of PTS2 proteins into the glycosome as RNAi experiments in *T. brucei* demonstrated that PEX5 and PEX7 are required for glycosome assembly [16].

*In vitro* studies clearly established that the *Leishmania* PEX7 recognized the prototype PTS2 signals on aldolase. Subcellular analysis of PEX7 in *L. donovani* promastigotes, revealed a dual cytosolic and glycosomal lumen distribution for this receptor. A similar bimodal distribution of PEX7 has also been observed in yeast and mammalian cells [9, 15, 28, 45] and suggests that PTS2 protein translocation involves the cycling of PEX7 in and out of the glycosome via a mechanism analogous to that proposed in yeast [45].

Collectively, these protein-protein interaction data suggest that PEX7 targets proteins into the glycosome via a model illustrated in Fig. 12. Nascent PTS2 polypeptides emerging from the ribosome are bound by the cytosolic *Leishmania* PEX7 receptor and the
resulting LdPEX7-PTS2 binary complex then binds to LdPEX5 preloaded with PTS1 and moves to the glycosomal surface where it docks on LdPEX14, or the LdPEX7-PTS2 migrates to the glycosome where it first binds to LdPEX14 and then recruits LdPEX5-PTS1. Based on in vitro interaction data, it is also possible that LdPEX5 and LdPEX7 may form a complex in the cytosol prior to becoming charged with the cargo proteins PTS1 and PTS2. However, the presence of PTS1 is not required for the Leishmania PEX7-PTS2 complex to associate with LdPEX14 as pull-down experiments using ldpex5NT, a fragment lacking PTS1 binding activity [10], precipitated LdPEX14, LdPEX7, and the PTS2 ligand aldolase. Once bound to LdPEX14, LdPEX5-PTS1, and LdPEX7-PTS2 appear to have distinctive fates. The PTS1 ligand is translocated into the glycosomal lumen while the empty LdPEX5 receptor is released back into the cytosol. Using a number of immunocytochemical assays, LdPEX5 has not been detected within the glycosome. This diverges from the models for PTS1 trafficking into the peroxisome where the PEX5 receptor functions via the extended shuttle mechanism [46, 47] or via the formation of a transient pore [24]. In contrast, the LdPEX7-PTS2 is imported into the glycosome and upon release of the PTS2 cargo, the LdPEX7 is exported back to the cytosolic compartment. The apparent accumulation of the Leishmania PEX7 in the glycosome suggests that the import of the LdPEX7-PTS2 complex may be a more facile process than the export of the empty LdPEX7 out of the glycosome.

A major feature distinguishing the glycosome PTS1 and PTS2 protein import machinery is the absence of a PEX13 homologue. Extensive searches of kinetoplastid parasite genome databases (www.genedb.org) using a number of search strategies have failed to identify a kinetoplastid PEX13 homologue. In higher eukaryotes, PEX13 interacts with PEX5, PEX7-PTS2, and PEX14 [21, 39, 48] and is critical for PTS1 and PTS2 protein targeting. PEX13 is proposed to trigger the release of PTS2 from PEX7 and participates in recycling PEX5 and PEX7 to the cytosol [12, 21, 25, 39]. The absence of a PEX13 homologue in kinetoplastid parasites suggests that protein translocation into the glycosome may operate by a different mechanism. Previous studies have demonstrated that the docking of LdPEX5-PTS1 to LdPEX14 on the glycosome surface decreases the LdPEX5-PTS1 binding affinity 10-fold which would permit the transfer of the PTS1
cargo to the docking/translocation complex and release of LdPEX5. However, the *Leishmania* PEX7 is postulated to enter the glycosome matrix bound to the PTS2 cargo and the empty *Leishmania* PEX7 receptor is proposed to shuttle out of the glycosome. Whether the *Leishmania* PEX7 depends on novel accessory molecules for protein import is not clear. Given that the glycosome is critical for parasite survival [5, 16], together with the fact that the glycosomal matrix protein import machinery appears to be functionally distinct and that the *Leishmania* and *Trypanosoma* peroxins share little sequence identity with the mammalian homologues, makes this biological pathway an attractive target for the development of novel antiparasitic agents.
Figure 12. Model of PTS1 and PTS2 protein import into *Leishmania* glycosomes. The schematic diagram illustrates a proposed model showing the protein-protein interactions between LdPEX5, LdPEX7, and LdPEX14 required for the import of PTS1 and PTS2 proteins into the glycosome. In *L. donovani*, newly-synthesized glycosomal proteins are trafficked to the glycosome by binding to the receptor proteins LdPEX5 and LdPEX7. In the cytosol, LdPEX5 and LdPEX7 may also form a complex prior to binding the PTS1 and PTS2 cargo proteins (1). Once loaded, the LdPEX5-PTS1 and LdPEX7-PTS2 complexes dock on LdPEX14-anchored glycosomal membrane. At the glycosome, the PTS1 cargo is delivered to the translocation complex and the LdPEX5 is released back into the cytosol (2). The LdPEX7-PTS2 complex is imported into the glycosomal matrix (3). Inside the glycosome, the LdPEX7-PTS2 complex dissociates and permits the shuttling of the LdPEX7 receptor to the cytoplasmic compartment (4). The thick arrow across the glycosomal membrane suggests that the import of the LdPEX7-PTS2 complex is more favorable than the export of the empty LdPEX7 receptor which results in an apparent accumulation of the receptor in the glycosome.
Chapter 3 References


Connecting Statement (Chapter 4)

The previous chapter dealt with the initial investigation of the role of *Leishmania* PEX7 in glycosomes. The following chapter involved a more detailed dissection of the mechanism of glycosome biogenesis by assessing the interactions between *Leishmania* PEX7 and other glycosomal components and by determining the protein complexes required for proper glycosomal protein sorting through various biochemical and biophysical techniques.
Chapter 4. The molecular dynamics of the *Leishmania* peroxin 7 with the components of glycosome biogenesis machinery

Ana Victoria C. Pilar, Rona Strasser, and Armando Jardim*

*Institute of Parasitology, McGill University, MacDonald Campus, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9*

* Manuscript in preparation for submission

**Abstract**

The *Leishmania* receptor protein PEX7 (LmPEX7) is involved in the sorting of PTS2 proteins for import into the glycosome. LmPEX7 interacts with the PTS1 receptor LdPEX5 and the membrane-associated protein LdPEX14. These interactions are mediated by distinct LmPEX7 binding domains on LdPEX5 and LdPEX14. In this study, we used a number of molecular and biophysical techniques to characterize the protein-protein interactions required for targeting PTS1 and PTS2 proteins into the glycosome. Structural analysis of LmPEX7 suggests that this protein forms a tetramer in solution but in the presence of a PTS2 ligand predominantly forms a tetramer. LmPEX7 consistently formed tetrameric structures in the presence of LdPEX5, LdPEX14 or PTS2 based on glycerol density gradient centrifugation further demonstrating the oligomeric structure of this PTS2 receptor. Using ELISA-based interaction assays, the interactions between LmPEX7 and other glycosomal components were quantitatively assessed. The interaction affinity between LmPEX7 and LdPEX5 is influenced by the presence of their respective cargo proteins suggesting that the formation of the LmPEX7-LdPEX5 complex is essential for the initial steps in PTS2 import and docking on LdPEX14. The subsequent release of the PTS2 cargo protein into the glycosomal matrix could be mediated by the LmPEX7-LdPEX14 interaction which modulates the affinity of LmPEX7 for its PTS2
ligand. The formation of protein complexes required for protein import corresponded with changes in the surface hydrophobicity, intrinsic fluorescence, and susceptibility to tryptic digestion of LmPEX7. Binding of LmPEX7 to other glycosomal proteins induced conformational changes in LmPEX7 that resulted in its increased susceptibility to tryptic digestion. Similarly, changes in intrinsic fluorescence and surface hydrophobicity in LmPEX7 were induced by binding LdPEX5 and PTS2. This study provided insight into the molecular dynamics of glycosomal protein trafficking into the glycosome which requires coordination between the two import pathways.
Introduction

Glycosomes are unique organelles found in the Family Trypanosomatidae which includes the medically important parasites *Trypanosoma* sp. and *Leishmania* sp., the causative agents of African sleeping sickness, Chagas disease, and leishmaniasis. Glycosomes are distantly related to peroxisomes in higher eukaryotes and share similar metabolic processes that include β-oxidation of fatty acids and ether lipid biosynthesis [1, 2]. Unlike peroxisomes, glycosomes lack the enzyme catalase, contain the first seven enzymes of glycolysis, and compartmentalize other pathways such as purine salvage, pentose phosphate pathway, and gluconeogenesis [2]. Glycosomes are important for the survival of the bloodstream form of *T. brucei* which is dependent on glycolysis for ATP production [2-4]. Because glycosomes and peroxisomes do not contain DNA or protein-synthesizing machinery, proteins targeted to these microbodies are imported into the matrix by two pathways called PTS1 and PTS2 [5]. These proteins are synthesized in the cytosol on free ribosomes and posttranslationally imported into the glycosomes or peroxisomes [2]. The proteins contain peroxisomal targeting signal sequences (PTS) either at the C-terminus (PTS1) or at the N-terminus (PTS2). The PTS1 signal denoted by the degenerate sequence serine-lysine-leucine (SKL) is found in many glycosomal and peroxisomal proteins and is recognized by the PTS1 import receptor peroxin 5 (PEX5) [6-9]. The PTS2 signal is a nonapeptide characterized by the sequence motif R/K-L/V/I-X₅-Q/H-L/A that is bound by the import receptor PEX7 [10-12]. Although the mechanism of protein import into peroxisomes and glycosomes has not yet been fully elucidated, various models have been proposed [13-15]. The major steps in protein translocation in yeast and mammalian cells involve the binding of the PTS ligands by the respective cytosolic receptors PEX5 and PEX7 to form a receptor-cargo complex, docking on the membrane through PEX14 or PEX13, translocation of the PTS-containing protein into the matrix, and recycling of the receptors back into the cytosol [16, 17]. The docking process involves the formation of a complex consisting of the peroxins PEX14, PEX13, and possibly PEX17 [18, 19] but how cargo proteins or receptors traverse the membrane is not known. One hypothesis that has been proposed is that the peroxisomal
PEX5 forms a transient pore on the peroxisomal membrane for the import of the PTS1 ligand [16, 19, 20]. After the PTS1 ligand is unloaded, the receptor is recycled back into the cytosol in an ATP-dependent manner mediated by the AAA+ proteins PEX1 and PEX6 [21, 22] and by a process of ubiquitination of PEX5 which involves PEX4, and the RING-finger proteins PEX2, PEX10, and PEX12 similar to E3 ubiquitin ligases [19, 23-25]. These downstream events result in the disassembly of the transient pore and relocation of PEX5 back into the cytosol. Import of PTS2 proteins through PEX7 is thought to occur via the same processes [16].

In yeast and mammalian peroxisomes, ~30 peroxins have been implicated in the biogenesis process, however, only ~15 of these peroxin homologs have been found and characterized in trypanosomatid glycosomes [26]. Sequence alignments of the *Leishmania* PEX5, PEX7, and PEX14 showed that these proteins have low sequence identities to their yeast, mammalian, and plant counterparts while a PEX13 homolog has not yet been identified [27-29]. Although it is tempting to suggest that similar processes of protein trafficking are at play in peroxisomes and glycosomes, this has yet to be determined.

The *Leishmania* PEX7 (LmPEX7) has been shown to have a dual localization in both the glycosomes and cytosol [29], while no appreciable amount of *L. donovani* PEX5 (LdPEX5) has been observed associated with the glycosomal membrane or inside the matrix, suggesting that a different mechanism of protein translocation could be taking place in the glycosomes [27, 29]. Moreover, previous studies have shown that the LdPEX14-LdPEX5 interaction is mediated by a novel binding motif on LdPEX5 [30] in contrast to that of the mammalian PEX14 binding site which requires the conserved pentapeptide repeats on the N-terminal part of PEX5 [31]. The binding of LdPEX5 to LdPEX14 modulates the LdPEX5-PTS1 binding affinity which is postulated to trigger the release of the cargo protein into the glycosomal matrix [32]. The LmPEX7 binding site on LdPEX5 does not share any homology to the PEX7 interaction domain found in the mammalian long isoform of PEX5 (PEX5L) or the redundant proteins PEX18 and PEX21 in yeast which are functionally similar to PEX5 [29, 33, 34].
The proper functioning of PEX5, PEX7, and PEX14 in trypanosomatids is essential for the survival of trypanosomatids as generation of PEX14 and PEX5 knockouts in *L. donovani* resulted in a conditional lethal phenotype [27, 28]. RNA interference of PEX5 and PEX7 in *T. brucei* caused the mis-targeting of PTS-containing proteins [27, 28, 35]. The interaction of these key proteins and sorting of the PTS proteins into glycosomes make the glycosome biogenesis machinery an attractive target for the development of drugs against trypanosomiasis and leishmaniasis [26, 36, 37].

In this study, the interactions and complex formation of LmPEX7, LdPEX5, and LdPEX14 in the presence or absence of PTS ligands were examined to understand the role of these proteins in glycosomal protein import and to determine how the PTS1 and PTS2 arms of the import pathways interact. These studies are important for gaining insight into the glycosome biology in *Leishmania.*
Materials and Methods

Expression and purification of recombinant proteins

Hexahistidine-tagged LmPEX7 (LmPEX7-His\textsubscript{6}) was expressed in \textit{E. coli} ER2566 (Novagen) as previously described [29] and purified under denaturing conditions by solubilizing inclusion bodies with 8.0 M urea in 40 mM Tris-HCl pH 8.0, 500 mM NaCl. The protein was refolded on a Ni\textsuperscript{2+}-NTA column using an 8.0 M – 0.5 M step down gradient of urea in buffer A (40 mM Tris-HCl pH 8, 500 mM NaCl, 0.05% Tween 20, 10 mM \textbeta-mercaptoethanol, 20% glycerol). Folded LmPEX7-His\textsubscript{6} was eluted with 320 mM imidazole in buffer A and exhaustively dialyzed against 40 mM Tris-HCl pH 8, 500 mM NaCl, 0.005% Tween-20, and 5% glycerol to remove imidazole and excess glycerol.

LdPEX14 (His\textsubscript{6}/S-LdPEX14) and ldald 1-77 (ldald 1-77-His\textsubscript{6}), a fragment containing residues 1-77 of \textit{L. donovani} aldolase, were purified as described previously [28, 29]. LdPEX5 and ldpex5 1-148, a fragment containing residues 1-148, were expressed as chitin fusion proteins as described previously [27, 29]. The \textit{L. donovani} xanthine phosphoribosyltransferase (LdXPRT) and xprt\textDelta AKL, a mutant lacking the PTS1 C-terminal signal, were purified by ion-exchange chromatography [27, 38].

In vitro binding assays

To determine the functional activity of the refolded LmPEX7, 20 \textmu g LdPEX14 was coupled to 50 \textmu l packed S-beads (Novagen) and incubated with 20 \textmu g refolded LmPEX7 or bovine serum albumin (BSA) in 50 \textmu l 40 mM sodium phosphate pH 8.0, 500 mM NaCl (PS) for 30 min at 20 °C with occasional mixing. Beads were washed 3 x 1.0 ml PS-1% Triton X-100 (PST) and 3 x 1.0 ml PS to remove unbound proteins. For pull-down assays to detect binding of native LdPEX7, detergent cell extracts of \textit{L. donovani} promastigotes were incubated with LdPEX14 affinity matrix. Cell extracts were prepared
by harvesting $1 \times 10^8$ mid-log promastigotes and washing 2 x 40 mM sodium phosphate pH 7.5, 150 mM NaCl (PBS). Cells were resuspended in 1 ml 40 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (TBST) and incubated for 15 min on ice. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was mixed with 50 µl packed LdPEX14 affinity beads. The mixture was incubated overnight at 4 °C with shaking. The beads were washed 3 x 1.0 ml TBST and 3 x 1.0 ml TBS. All bound proteins were resolved in SDS-PAGE and analyzed by Western blot using anti-LmPEX7 antisera.

**Gel permeation chromatography**

Gel permeation chromatography was performed using a Beckman-Coulter HPLC system equipped with a 300 x 7.8 mm Bio-Sil SEC 250 column (Bio-Rad) equilibrated with 40 mM sodium phosphate pH 7.5, 150 mM NaCl, 20% glycerol, and 5 mM β-mercaptoethanol at a flow rate of 0.5 ml/min. 20 µg of LmPEX7 or a mixture of LmPEX7 and Ldald 1-77 at a molar ratio of 1:1 or 1:2 was injected and the column eluant was detected at 280 nm. Fractions were collected and the proteins were precipitated with 15% trichloroacetic acid (TCA) and analyzed by Western blot using anti-LmPEX7 antibodies. The standards used to calibrate the column were thyroglobulin (670 kDa), bovine immunoglobulin G (IgG) (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa) (Bio-Rad).

**LmPEX7 cross-linking**

10 µM LmPEX7 previously dialyzed in PBS containing 5% glycerol was treated with 0.02-0.5% glutaraldehyde and incubated at 25 °C for 30 min. The reaction was terminated by adding 25 mM Tris-HCl pH 7.5 and incubated for 15 min. The cross-linked and non-cross-linked proteins were analyzed by Western blot using anti-LmPEX7 antisera.
The structure of the LmPEX7-LdPEX5, LmPEX7-Ildald 1-77, and LmPEX7-LdPEX14 complexes were analyzed by mixing 1:1 molar ratios of the recombinant proteins (200 μl reaction) and incubating for 15 min on ice. The mixtures were overlayed on a 5-35% glycerol gradient in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 100 mM MgCl, and protease inhibitor cocktail (Roche). The gradient was centrifuged at 36,000 rpm for 18 h at 4 °C using an SW41 Beckman rotor. 500 μl fractions were obtained by puncturing the bottom of the centrifuge tube and the proteins were precipitated by trichloroacetic acid (TCA). The proteins were analyzed by Western blot using anti-LmPEX7, anti-LdPEX5, and anti-LdPEX14 antisera.

**ELISA binding assays**

For the LmPEX7-LdPEX5 interaction, high-binding flat-bottom 96-well microtiter plates (Perkin-Elmer) were coated with 1 μg LdPEX5 in 100 μl PBS (40 mM sodium phosphate pH 7.5, 150 mM NaCl) for 16 h at 4 °C. Plates were blocked with 2% bovine serum albumin (BSA) (Sigma) in PBS-0.1% Tween 20 (PBST) for 1 h at 25 °C then incubated with increasing concentrations of LmPEX7 (0.5 - 1000 nM) in 100 μl PBST/2% BSA for 2 h at 25 °C in the presence or absence of LdPEX14, LdXPRT or Ildald 1-77 at a constant concentration of 250 nM. Bound LmPEX7 was quantified by indirect ELISA using guinea pig anti-LmPEX7 antisera (1:10,000) and donkey anti-guinea pig horseradish peroxidase-conjugated (HRP) secondary antibody (1:10,000) (Cedarlane) in PBST/2% BSA. As a control for non-specific binding, secondary antibodies alone were used or plates coated with LdXPRT (1 μg/100 μl PBS) were incubated with LmPEX7 (0.5 - 1000 nM), and probed with anti-LmPEX7 antisera. As an additional control, wells coated with LdPEX5 (1 μg/100 μl PBS) were incubated with Ildald 1-77 (0.5-850 nm) and probed with anti-His tag antisera.
To examine the LmPEX7-LdPEX14 and LmPEX7-Ldald 1-77 interactions, microtiter plates were coated with 1 µg LdPEX14 or Ldald 1-77 in 100 µl PBS then incubated with increasing concentrations of LmPEX7 (0.5 - 1000 nM) in the presence or absence of a constant concentration of 250 nM LdPEX5, LdPEX14 or Ldald 1-77. Bound LmPEX7 was detected with anti-LmPEX7 antisera. As a control for non-specific binding, secondary antibodies alone were used or plates coated with Ldald 1-77 (1 µg/100 µl PBS) were incubated with LdPEX5 or LdPEX14 (0.5 - 850 nM), and probed with anti-LdPEX5 or anti-LdPEX14 antisera.

Similar experiments were performed to examine the LdPEX5-LdPEX14 and LdPEX5-LdXPRT interactions in the presence of LmPEX7. LdPEX5-coated plates (1 µg/100 µl PBS) were incubated with increasing concentrations of either LdPEX14 or LdXPRT (0.4 - 850 µM) in the presence or absence of a constant concentration of 0.25 µM LmPEX7. Bound LdPEX14 or LdXPRT were quantified by probing with polyclonal LdPEX14-specific rabbit antisera (1:20,000), rabbit anti-LdXPRT antibody (1:2,000), and donkey anti-rabbit HRP secondary antibody (1:10,000) (GE Healthcare).

All experiments were done in triplicate in 3-6 independent trials. Plates were developed with the chromogenic substrate 2,2’-azonobis-3-ethylbenzthiazoline-sulfonic acid (ABTS). Data analysis was performed using the ORIGIN 7.0 software (Microcal Software).

**Tryptic digestion**

The conformational changes induced by the LmPEX7-LdPEX5 or LmPEX7-LdPEX14 complexes were examined by mixing 100 µg LmPEX7 and LdPEX5 or LdPEX14 (1:1 molar ratio) in 120-180 µl TBS (40 mM Tris-HCl pH 8, 150 mM NaCl) were treated with porcine trypsin (Promega) (1:200 trypsin:substrate) and incubated at 25 °C. 10-15 µl aliquots were removed at various time points and the reaction was terminated by adding 5 µl EDTA-free protease inhibitor cocktail (Roche), incubated on ice for 20 min, and mixed with an equal volume of 2X Laemmli SDS-PAGE sample buffer. Proteins were
analyzed by Western blot using anti-LmPEX7 and anti-LdPEX5 antisera. Bands corresponding to parent and digested LmPEX7 and LdPEX5 molecules were quantified by densitometry using the ImageJ software [39].

The effect of PTS1 or PTS2 on the proteolytic degradation kinetics of LmPEX7, LdPEX5 or LdPEX14 was determined by adding a 10-fold molar excess of LdXPRT or ldald 1-77 to the protein complexes prior to tryptic digestion.

**Western blot analysis and description of antibodies**

Proteins were resolved on an 8% or 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane (PVDF) (Bio-Rad). Membranes were blocked with 3% skim milk in TBST (40 mM Tris-HCl pH 8, 150 mM NaCl, and 0.01% Tween-20) for 1 h at 25 °C, incubated with primary antibody for 1 h at 25 °C in TBST-3% milk, then incubated with horseradish peroxidase-conjugated (HRP) secondary antibody for 45 min at 25 °C. Blots were developed using Western Lightning® chemiluminescence reagent (Perkin-Elmer). Primary antibodies used in Western blot analysis and ELISA were polyclonal guinea pig anti-LmPEX7 antibodies (1:10,000) which were raised against LmPEX7-His<sub>6</sub>; polyclonal rabbit anti-LdPEX5 antisera (1:10,000) which were raised against the recombinant LdPEX5; polyclonal rabbit anti-LdPEX14 antisera (1:20,000) which specifically recognize the first 63 amino acid residues at the N-terminus of His<sub>6</sub>/S-LdPEX14; polyclonal rabbit anti-LdXPRT (1:10,000); and mouse anti-His tag antisera (1:10,000) (GE Amersham). Custom antibodies for LmPEX7, LdPEX5, and LdPEX14 were obtained from Cocalico Biologicals. IgG HRP-conjugated secondary antibodies used were donkey anti-guinea pig (1:10,000) (Cedarlane), donkey anti-rabbit (1:10,000) (GE Healthcare) or goat anti-mouse (Cedarlane).
**Fluorescence measurements**

Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a thermostatically-controlled cell holder and cells with 10 mm optical path length, and 5 nm excitation and emission slit widths. LmPEX7 was diluted in 40 μl TBS to a final concentration of 20 μM and fluorescence spectra measurements were performed at 25 °C using an excitation wavelength of 295 nm. Emission was recorded from 300-450 nm at a rate of 120 nm/min and spectra were presented as mean values of five measurements. Baseline spectra were subtracted from the sample readings and data analysis was performed using ORIGIN 7.0 software (Microcal Software).

For titration experiments, 20 μM LmPEX7 was mixed with increasing concentrations (1:0.2, 1:0.5, 1:1 molar ratios) of ldalδ 1-77 or ldpe5 1-148 and changes in the intrinsic fluorescence of LmPEX7 were measured. Baseline spectra from only ldalδ 1-77 or ldpe5 1-148 in TBS were subtracted from the sample readings.

To probe for the presence of hydrophobic surfaces of LmPEX7, 20 μM LmPEX7 in TBS was mixed with 50 μM 8-anilino-1-naphthalenesulfonic acid (ANS) and samples were excited using a wavelength of 375 nm and the emission spectra were recorded from 400-600 nm. For fluorescence resonance energy transfer experiments, an excitation wavelength of 295 nm was used and emission spectra were collected from 300 to 600 nm. Titration experiments in the presence of ANS were performed by adding increasing concentrations of ldalδ 1-77 (1:0.05-1:3 molar ratios) or ldpe5 1-148 (1:0.2, 1:0.5, and 1:1 molar ratios). Baseline fluorescence for ANS or ANS with ldalδ 1-77 or ldpe5 1-148 were subtracted from the sample readings.

Fluorescence quenching experiments were performed by titrating 20 μM LmPEX7 in TBS with increasing concentrations (5 mM – 600 mM) of potassium iodide (KI) or acrylamide and changes in fluorescence intensity changes measured using an excitation
wavelength of 295 nm and recording the emission spectra from 300-450 nm. Results were analyzed using the Stern-Volmer relationship for steady-state collisional quenching, \( F_0/F = 1 + K_{SV}[Q] \), where \( F_0 \) is the fluorescence intensity in the absence of a quencher, \( F \) is the fluorescence intensity in the presence of a quencher, \( K_{SV} \) is the Stern-Volmer constant, and \( Q \) is the quencher concentration [40]. The \( K_{SV} \) was determined by linear regression from a plot of the quencher concentration versus \( F_0/F \) [41].
Results

Quaternary structure of LmPEX7

It was previously reported that the soluble form of recombinant LmPEX7 expressed in *E. coli* co-purified with GroEL and attempts to dissociate the chaperone using various techniques were not successful. The presence of this chaperonin, however, did not affect the capacity of LmPEX7 to bind LdPEX5, LdPEX14 or PTS2-containing proteins aldolase and hexokinase [29]. To generate a form of LmPEX7 devoid of GroEL, LmPEX7 was extracted from inclusion bodies under denaturing conditions using 8 M urea and purified on Ni²⁺-NTA column. The protein was refolded on the column using a gradual step down gradient of urea to a concentration of 0.5 M. The residual urea was removed by washing the column with TBS containing 20% glycerol as a chemical chaperone and eluting LmPEX7 with 320 mM imidazole in TBS/20% glycerol. The refolded LmPEX7 remained soluble even with 5% glycerol (Fig. 13A). Characterization using a series of pull-down assays with LdPEX14 showed that the refolded LmPEX7 (Fig. 13B, lane 4) was functionally active and bound LdPEX14 similar to the native LdPEX7 (Fig. 13B, lane 1). As a control, BSA was not precipitated by LdPEX14 (Fig. 13B, lane 2) and the anti-LmPEX7 antibodies were specific to LmPEX7 as no bands were detected in the control set-up containing LdPEX14 affinity beads incubated with buffer alone (Fig. 13B, lane 3). Similar results were obtained from pull-down assays using IdalD 1-77 and IdpexNNT affinity matrices (data not shown).

Analysis of the refolded LmPEX7 on a Bio-Sil GPC column using a mobile phase containing 20% glycerol revealed that this protein migrated as a potential doublet with apparent molecular masses of ~83 and ~120 kDa, sizes consistent with this protein being in equilibrium between a dimeric and tetrameric form (Fig. 13C). Surprisingly, GPC analysis of mixtures containing either 1:1 or 1:2 molar ratio of LmPEX7 and IdalD 1-77, a small PTS2 ligand protein, showed the loss of the shoulder peak at ~ 83 kDa and suggested that the PTS2 ligand stabilized the tetrameric form LmPEX7 (Fig. 13C).
contrast, the human PEX7 has been reported to form a dimer [42] or a monomer [43]. However, only the monomeric form was observed to bind the PTS2 signal.

To assess whether the changes observed with the PTS2 ligand were due to an altered quaternary structure or changes in the hydrodynamic volume, LmPEX7 was analyzed by cross-linking and glycerol density gradient centrifugation. In the presence of increasing concentrations of glutaraldehyde, LmPEX7 formed dimeric and tetrameric structures consistent with data from gel permeation studies (Fig. 13D). The quaternary structure of LmPEX7 was further examined by glycerol density gradient centrifugation, a method that circumvents problems with changes in hydrodynamic volume of a protein in GPC. LmPEX7 alone or in complex with LdPEX5, LdPEX14, and ldald 1-77 consistently migrated as a tetramer in the gradient (Fig. 14A, panels i-iv). Interestingly, LdPEX5 alone migrated as a monomer but co-sedimented with LmPEX7 in the middle portion of the gradient suggesting a tetramer-monomer complex of LmPEX7 and LdPEX5 (Fig. 14A, panels v-vi). Similar to previous reports [32], LdPEX14 migrated as a multimeric complex of about >900 kDa (Fig. 14A, panel vii) which did not co-migrate with LmPEX7 suggesting that the LmPEX7-LdPEX14 quaternary complex may not be stable or is transient in the absence of either LdPEX5 or ldald 1-77.
Figure 13. Structure analysis of LmPEX7. (A) LmPEX7 was purified under denaturing conditions and refolded on a Ni$^{2+}$-NTA column using a step-down urea gradient. SDS-PAGE analysis of the purified LmPEX7 showed a homogeneous band. (B) In vitro pull-down assay using LdPEX14 S-beads incubated with detergent cell extracts of *L. donovani* promastigotes (*lane 1*) and refolded LmPEX7 (*lane 4*). Bound proteins were analyzed by Western blot using anti-LmPEX7. Bovine serum albumin (BSA) (*lane 2*) or TBS (*lane 3*) was incubated with the beads to detect non-specific binding. (C) The native structure of LmPEX7 (*black line*) and LmPEX7-ldald 1-77 complex (1:1 mole ratio) (*gray line*) were analyzed by gel permeation chromatography. The collected fractions were analyzed by Western blot using anti-LmPEX7. (*Inset*) Fractions corresponding to the LmPEX7 peak (*black line*). The column was calibrated with thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B$_{12}$ (1.35 kDa). (D) 10 µM of LmPEX7 in PBS was mixed with 0.02-0.5% glutaraldehyde 30 min at 25 °C and reactions were terminated by the addition of 25 mM Tris-HCl pH 7.5. Proteins were analyzed by Western blot using anti-LmPEX7. Arrows show the dimeric and tetrameric forms of LmPEX7.
Figure 14. Glycerol density gradient centrifugation of protein complexes. (A) 50 μg of LmPEX7 was mixed with LdPEX5, ldald 1-77 or LdPEX14 at 1:1 molar ratio and the protein complexes were overlayed on a 5-35% glycerol gradient and fractionated at 36,000 rpm for 18 h at 4°C. Fractions were analyzed by Western blot using anti-LmPEX7 (panels i-iv), anti-LdPEX5 (panels v-vi), and anti-LdPEX14 (panel vii) antisera. The molecular mass of the protein complexes were calibrated by running separate glycerol density gradients containing the standards thyroglobulin (670 kDa), bovine immunoglobulin G (IgG) (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and human immunoglobulin M (IgM) (900 kDa).
**LmPEX7 interactions**

Previous studies showed that LmPEX7 bound LdPEX5 and LdPEX14 through a domain localized to amino acids 111-148 and 120-148 on LdPEX5 and LdPEX14, respectively [29]. To quantitatively assess the binding of LmPEX7 to LdPEX5, LdPEX14 or PTS2, an ELISA-based assay was employed. Immobilizing LdPEX5 on microtiter plates and incubating with increasing concentrations of LmPEX7 (Fig. 15A) showed saturable binding and gave a dissociation constant (K_D) value of 114 nM (Table 1). However, this interaction was decreased by 20-fold when LdPEX5 bound to microtiter plates was loaded with the PTS1 ligand LdXPRT (Fig. 15A, Table 1). These results indicate that the PTS1 ligand either triggered a conformational change in LdPEX5 that sequestered the LmPEX7 binding site or that it interfered sterically. A similar decrease in binding affinity was observed with a peptide containing the PTS1 signal alanine-lysine-leucine (AKL) [27] suggesting the possibility that these effects were not due to a steric effect associated with the binding of the PTS1 ligand (data not shown). In contrast, loading LmPEX7 with the PTS2 fragment ldald 1-77 increased the LmPEX7-LdPEX5 binding affinity by ~3 fold (Table 1). Interestingly, the LmPEX7-LdPEX5 interaction was not altered by LdPEX14 (Fig. 15A). This was surprising since LmPEX7 has been shown to form tertiary complexes with LdPEX5 and LdPEX14 [29]. Control experiments showed no detectable binding of LmPEX7 to LdXPRT or LdPEX5 to ldald 1-77. For all control set-ups, the average absorbance values were less than 0.1 (indicated in Fig. 15). Moreover, no cross-reactivity of the LmPEX7 antisera with LdPEX5, LdPEX14 or LdXPRT was observed.

Analysis of the LmPEX7-PTS2 interactions revealed that LmPEX7 bound the PTS2 ldald 1-77 with a K_D of ~23 nM (Fig. 15B, Table 1) and this binding affinity was not altered by LdPEX5 (Fig. 15B). That LdPEX5 bound to the LmPEX7-ldald 1-77 complex was confirmed by probing these complexes with anti-LdPEX5 antibodies. In addition, pull-down assays showed that an LdPEX5 affinity matrix could precipitate not only the native
*Leishmania* PEX7 but also PTS2 proteins [29] In contrast, the binding of PTS2 to LmPEX7 dramatically decreased in the presence of LdPEX14 (Fig. 15B).

To corroborate the binding affinities, alternative combinations using different substrates anchored to the solid support were investigated. Immobilizing LdPEX5 on the microtiter plates gave $K_D$ values of 16 nM and 15 nM for the LdPEX5-LdXPRT and LdPEX5-LdPEX14 interactions, respectively (Table 2, Fig. 15C & 15D). The presence of LmPEX7 did not affect the binding affinities of these interactions.

Attempts to examine the LmPEX7-LdPEX14 interaction were complicated by the fact that saturation kinetics could not be achieved (Fig. 15E) suggesting that the interaction of LmPEX7 with LdPEX14 is relatively weak.
Table 1. Equilibrium dissociation constants for LmPEX7-LdPEX5, LmPEX7-ldald 1-77, and LmPEX7-LdPEX14 interactions.

The first column denotes the immobilized protein on plates incubated with the ligand LmPEX7 in the presence or absence of another ligand or modulator.

<table>
<thead>
<tr>
<th>Immobilized protein</th>
<th>Ligand</th>
<th>Modulator</th>
<th>(K_D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LdPEX5</td>
<td>LmPEX7</td>
<td>LdPEX14</td>
<td>114 ± 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ldald 1-77</td>
<td>125 ± 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LdXPRT</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>ldald 1-77</td>
<td>LmPEX7</td>
<td>LdPEX5</td>
<td>35 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LdPEX14</td>
<td>&gt;303</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LdXPRT</td>
<td>&gt;66</td>
</tr>
<tr>
<td>LdPEX14</td>
<td>LmPEX7</td>
<td>LdPEX5</td>
<td>&gt;329</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ldald 1-77</td>
<td>&gt;323</td>
</tr>
</tbody>
</table>

Table 2. Equilibrium dissociation constants for the LdPEX5-LdPEX14 and LdPEX5-LdXPRT interactions in the presence or absence of LmPEX7.

<table>
<thead>
<tr>
<th>LdPEX5</th>
<th>+ LmPEX7</th>
</tr>
</thead>
<tbody>
<tr>
<td>LdPEX14</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>LdXPRT</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>
Figure 15. Determination of equilibrium dissociation constants by ELISA-based protein interaction assays. (A) Microtiter plates coated with LdPEX5 were incubated with increasing concentrations of LmPEX7 (■) in the presence or absence of LdPEX14 (○), LdXPRT (▲), or Idald 1-77 (▽). Bound LmPEX7 was quantified by indirect ELISA using anti-LmPEX7. The measured absorbance values were normalized and the averages plotted against the log of the LmPEX7 concentration using the Microcal Origin 7.0 software. As a control, immobilized LdPEX5 was incubated with Idald 1-77 alone and probed with anti-His tag antisera. The average absorbance value was 0.0547 ± 0.0004. (B) For the LmPEX7-Idald 1-77 interactions, microtiter plates coated with the
PTS2 ligand ldald 1-77 were incubated with increasing concentrations of LmPEX7 (■) in the presence or absence of LdPEX5 (○) or LdPEX14 (▼). As a control, immobilized ldald 1-77 was incubated with LdPEX5 or LdPEX14 and probed with anti-LdPEX5 or anti-LdPEX14 antisera. The average absorbance values were 0.075 ± 0.024 and 0.06 ± 0.02, respectively. (C) For the LdPEX5-LdPEX14 interactions, wells coated with LdPEX5 were incubated with increasing concentrations of LdPEX14 (■) in the presence or absence of LmPEX7 (○). Bound LdPEX14 was quantified using anti-LdPEX14 antisera. (D) For the LdPEX5-LdXPRT interactions, wells coated with LdPEX5 were incubated with increasing concentrations of the PTS1 ligand LdXPRT (■) in the presence or absence of LmPEX7 (○). Bound LdXPRT was quantified using anti-LdXPRT. As a control, wells coated with LdXPRT were incubated with LmPEX7 and probed with anti-LmPEX7 antisera. The average absorbance value was 0.063 ± 0.025. (E) For the LmPEX7-LdPEX14 interactions, microtiter plates coated with LdPEX14 were incubated with increasing concentrations of LmPEX7 (■) in the presence or absence of LdPEX5 (○) or ldald 1-77 (▼). Bound LmPEX7 was quantified using anti-LmPEX7. All experiments were performed in triplicate in 3-6 independent trials.
Affinity purification experiments using nonionic detergent extracts from *L. donovani* promastigotes revealed that LdPEX5 formed a ternary complex with LdPEX7 and LdPEX14 [29]. The conformational changes induced by protein complex formation were assessed by limited tryptic proteolysis. Tryptic digests of LmPEX7 alone or LmPEX7 loaded with ldald 1-77 showed similar digestion patterns suggesting that no major conformational changes of LmPEX7 was required for PTS2 binding (Fig. 16A *panel i*, Fig. 16B, Table 3). Moreover, since LmPEX7 contains 29 predicted trypsin cleavage sites, the relative resistance to proteolysis indicates that LmPEX7 has a relatively compact structure. In the presence of a PTS2 ligand, LmPEX7 showed an increased susceptibility to proteolysis with $t_{1/2}$ of ~60 min (Fig. 16A *panel ii*, Fig. 16B, Table 3). In contrast, binding LdPEX5 or LdPEX14 alone or in combination induced a conformational change that markedly increased the degradation of LmPEX7 and resulted in a $t_{1/2}$ of ~10-15 min (Fig. 16A *panels iii, vi*, Fig. 16B, Table 3). Degradation of LmPEX7 was further accelerated in the presence of LdPEX5, PTS1, and PTS2 ligands (Fig. 16A *panels iv, v, ix*, Fig. 16B) indicating that these interacting proteins triggered conformational changes in LmPEX7 rendering this protein susceptible to proteolysis. Similarly, binding of LdPEX14 to LmPEX7 loaded with ldald 1-77 also decreased the $t_{1/2}$ of LmPEX7 to 5 min (Fig. 16A *panel viii*, Fig. 16B).

To analyze the effect of LmPEX7 on LdPEX5, the digest mixtures were probed with anti-LdPEX5 antisera. Tryptic digestion of LdPEX5 alone resulted in the production of a ~30 kDa fragment that was resistant to proteolysis. The $t_{1/2}$ for this degradation was estimated to be ~15 min (Table 3). Previous studies have reported that the ~30 kDa fragment corresponded to a C-terminal domain spanning residues 303-625 [27]. In the presence of LmPEX7 and LdXPRT, LdPEX5 was degraded within 5 min (Fig. 16C & 16D, Table 3). Similarly, the presence of LmPEX7, LdPEX14, and LdXPRT caused LdPEX5 degradation within 10 min (Fig. 16D, Table 3). In all cases, the 30 kDa fragment
remained intact suggesting these interactions were causing structural changes at the N-terminal portion of LdPEX5.

In the absence of other proteins, LdPEX14 was degraded within 7 min (Fig. 16E & 16F, Table 3). The LdPEX14 digestion profile remained relatively unchanged upon binding LmPEX7 (Fig. 16E & 16F) with a $t_{1/2}$ estimated to be 5 min (Table 3). The binding of LdPEX5 alone, LdPEX5-LdXPRT or LmPEX7-Ldald 1-77 complex caused a rapid degradation of LdPEX14 with complete loss observed within 2-5 min (Fig. 16E & 16F, Table 3). This suggests that the docking of the LdPEX5-PTS1 or LmPEX7-Ldald 1-77 complexes triggered notable conformational changes in LdPEX14. Since the anti-LdPEX14 antisera specifically recognize the first 63 amino acid residues at the N-terminus of LdPEX14, the tryptic digestion also showed that the C-terminal region of LdPEX14 is more susceptible to proteolysis (Fig. 16E).

The digestion profiles of the LdPEX5-LdXPRT complex in the presence or absence of LmPEX7 showed no difference in proteolysis of LdXPRT (Fig. 16G).
Table 3. Half-life ($t_{1/2}$) of LmPEX7, LdPEX5, and LdPEX14 protein complexes in limited proteolytic digestion using trypsin.

<table>
<thead>
<tr>
<th>Protein complex</th>
<th>t$_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LmPEX7</td>
</tr>
<tr>
<td>LmPEX7 alone</td>
<td>&gt;15</td>
</tr>
<tr>
<td>LdPEX5 alone</td>
<td>-</td>
</tr>
<tr>
<td>LdPEX14 alone</td>
<td>-</td>
</tr>
<tr>
<td>LmPEX7 +</td>
<td></td>
</tr>
<tr>
<td>ldald 1-77</td>
<td>~60</td>
</tr>
<tr>
<td>ldald 1-77 + LdPEX5</td>
<td>5</td>
</tr>
<tr>
<td>LdPEX5</td>
<td>15</td>
</tr>
<tr>
<td>LdPEX5 + LdXPRT</td>
<td>&lt;2</td>
</tr>
<tr>
<td>LdPEX14</td>
<td>10</td>
</tr>
<tr>
<td>LdPEX14 + LdPEX5</td>
<td>10</td>
</tr>
<tr>
<td>LdPEX14 + ldald 1-77</td>
<td>5</td>
</tr>
<tr>
<td>LdPEX14 + LdPEX5 + LdXPRT</td>
<td>5</td>
</tr>
<tr>
<td>Time (min)</td>
<td>LmPEX7 *</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
</tr>
</tbody>
</table>

**A**

<table>
<thead>
<tr>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
</tr>
</tbody>
</table>

**B**

![Graph showing percent of normal over time for different conditions](image)

**C**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>LdPEX5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>180</td>
<td>25</td>
</tr>
</tbody>
</table>

**D**

![Graph showing percent of normal over time for different conditions](image)
Figure 16. Tryptic digestions of protein complexes. Changes in the digestion profiles of glycosomal proteins during complex formation were monitored by tryptic digestion. Protein complexes were treated with trypsin (1:200 trypsin:substrate) and incubated at 25 °C. Aliquots were taken at various time points and reactions were terminated by adding protease inhibitor cocktail and SDS-PAGE sample buffer. Protein digests were analyzed by Western blot using anti-LmPEX7, anti-LdPEX5, anti-LdPEX14, and anti-LdXPRT. (A) Tryptic digestion profiles of LmPEX7 alone (i), LmPEX7 with PTS2 ligand ldald 1-77 (ii), LdPEX5 (iii), LdPEX5 + ldald 1-77 (iv), LdPEX5 + LdXPRT (v), LdPEX14 (vi), LdPEX14 + LdPEX5 (vii), LdPEX14 + ldald 1-77 (viii), and LdPEX14 + LdPEX5 + LdXPRT (ix). Results were analyzed using the ImageJ software and presented as a plot of the digestion time versus the percent ratio of densitometry measurements for undigested and digested bands (B, D, F). (C) Digestion patterns of LdPEX5 alone and LdPEX5 mixed with LdXPRT, LmPEX7, LmPEX7 + LdXPRT, LmPEX7 + ldald 1-77, LdPEX14 + LmPEX7 + LdXPRT, and LmPEX7 + LdPEX14. (E) Tryptic digests of LdPEX14 alone and complexed with LdPEX5 + LmPEX7, LmPEX7, LmPEX7 + LdPEX5 + LdXPRT, and LmPEX7 + ldald 1-77. (G) Tryptic profile of LdXPRT complexed with LdPEX5 and LdPEX5 + LmPEX7.
Intrinsic fluorescence analysis of LmPEX7

LmPEX7 contains 10 tryptophan residues, six of which are part of the conserved WD40 motifs found in this receptor [29]. Analysis of the fluorescence spectra using an excitation wavelength of 295 nm which is specific for tryptophan residues revealed a complex spectra with two emission maxima at 330 $\lambda_{\text{max}}$, diagnostic of tryptophan residues located in a buried nonpolar environment, and at 366 nm typically observed for tryptophan residues in an aqueous polar environment (Fig. 17A & B). The presence of increasing concentrations of the PTS2 ligand ldald 1-77 doubled the fluorescence intensity of LmPEX7 confirming that the binding of PTS2 triggered a structural change in LmPEX7 (Fig. 17A). ldald 1-77 alone did not have significant fluorescence emission as this fragment does not contain any tryptophan residues. A two-fold increase in intensity was observed in the presence of ldpex5 1-148, a fragment of LdPEX5 that contains the binding domain for LmPEX7 (Fig. 17B) [29]. Adding ldald 1-77 to the LmPEX7-ldpex5 1-148 did not alter the fluorescence intensities (data not shown). These results suggest that the binding of PTS2 or ldpex5 1-148 caused conformational changes in LmPEX7 that altered the configuration of one or more tryptophan residues resulting in increased fluorescence intensities.
Figure 17. Fluorescence measurements. Fluorescence spectra were carried out at 25 °C using an excitation wavelength of 295 nm and recording emission at 300-450 nm. Spectra were presented as mean values of five measurements and baseline fluorescence values were subtracted from sample readings. Results were analyzed using the ORIGIN 7.0 software. (A) Intrinsic fluorescence of 20 μM LmPEX7 in TBS (solid black line) and in the presence of increasing concentrations (1:0.5, 1:1, 1:4 molar ratios) of ldald 1-77 (dashed, dotted, and gray lines). (B) Fluorescence intensities of LmPEX7 alone (solid black line) and in the presence of increasing concentrations (1:0.1, 1:0.2, 1:0.5, and 1:1 molar ratios) of ldpx5 1-148 (dashed, dotted, and gray lines). Fluorescence intensity values are presented in arbitrary units (a.u.).
The tight binding of GroEL to LmPEX7 reported in previous studies suggests that LmPEX7 contained a potentially exposed hydrophobic surface promoting the tight binding of this chaperone [29]. To examine this hypothesis and assess the effect of LmPEX7 binding PTS2 or LdPEX5, fluorescence measurements were performed using the environment sensitive fluorescent probe ANS. In a polar solution, this probe is relatively non-fluorescent. However, on binding a hydrophobic region on a protein, a marked increase in fluorescence intensity and blue shift in the emission wavelength is observed [44]. The fluorescence intensity of ANS increased 200-fold after binding LmPEX7 with a corresponding shift in the emission from 510 nm to 480 nm (Fig. 18A & B). The addition of ldald 1-77 to LmPEX7 did not significantly alter the ANS fluorescence (Fig. 18A) indicating that the PTS2 ligand did not induce structural changes in LmPEX7 that shrouded the hydrophobic region or that the binding of PTS2 was occurring at the hydrophobic surface. These data support results from tryptic digestions of LmPEX7. Control experiments showed ldald 1-77 alone caused only minor increases in the ANS fluorescence (Fig. 18A). In contrast, addition of ldpex5 1-148, a fragment containing the LmPEX7 binding domain, caused a 30% increase in ANS fluorescence (Fig. 18B) suggesting that the binding of this fragment induced a conformational change in LmPEX7 that increased the exposure of the hydrophobic domain. This conformational change corresponds to the increased susceptibility of LmPEX7 to proteolysis. Interestingly, addition of ldald 1-77 dramatically decreased the ANS fluorescence. This decrease is not likely due to the displacement of ldpex5 1-148 since previous pull-down assays using an LdPEX5 affinity matrix precipitated both the Leishmania PEX7 and PTS2 ligands [29]. Alternatively, ldald 1-77 is binding to a region on LmPEX7 exposed by ldpex5 1-148 and displacing the ANS probe.

Excitation of LmPEX7 in the presence of ANS at 295 nm showed a marked quenching of its intrinsic fluorescence due to resonance energy transfer from the tryptophan residues to ANS indicating that some of these residues are found in hydrophobic environments (Fig. 18C) [45]. Adding ldald 1-77 to LmPEX7 did not prevent the quenching by ANS which
suggests that the PTS2 binding site is situated further from regions containing the accessible tryptophanyl residues located in the hydrophobic pockets (Fig. 18C).
Figure 18. Changes in surface hydrophobicity. LmPEX7 was mixed with 8-anilino-1-naphthalenesulfonic acid (ANS) and titrated with increasing concentrations of (A) ldlal 1-77 and (B) ldpex5 1-148. Fluorescence was measured at 25°C using a 375 nm excitation wavelength and the emission collected at 380-600 nm. (A) Fluorescence spectra of LmPEX7 alone (thin black line), LmPEX7 with ANS (solid black line), LmPEX7 with ldlal 1-77 at 1:0.2, 1:0.5, 1:1 mole ratios (dashed, dotted, solid gray lines), and ldlal 1-77 with ANS (dashed light gray line). (B) LmPEX7 alone (thin black line), LmPEX7 with ANS (solid black line), and LmPEX7 with ldpex5 1-148 at 1:0.2, 1:0.5, and 1:1 mole ratios (dashed and dotted lines). ldlal 1-77 added to the LmPEX7-ldpex5 1-148 complex reduced the surface hydrophobicity of LmPEX7 (gray line). (C) Fluorescence spectra of LmPEX7 quenched by ANS (gray line). Measurements were done at 295 nm excitation and emission collected from 300-600 nm. The addition of ldlal 1-77 to the mixture did not abrogate quenching of LmPEX7 (dashed and dotted lines). Fluorescence intensity values are presented in arbitrary units (a.u.).
**Fluorescence quenching**

Fluorescence quenching experiments were performed to describe the environment and solvent polarity of the tryptophan residues of LmPEX7 and to provide structural information about the conformation of this protein. The steady-state collisional quenching based on the Stern-Volmer equation assumes that there is one fluorophore present in a single environment and is accessible to the quencher [41]. The emission of an exposed tryptophan residue strongly decreases in the presence of a quencher giving a $K_{SV}$ of $\sim 8 \text{ M}^{-1}$ [41]. The plot of the quenching of LmPEX7 by KI and acrylamide based on the classical Stern-Volmer equation was linear with a $K_{SV}$ of 1.4 M$^{-1}$ and 1.8 M$^{-1}$, respectively (Fig. 19). This suggests that the tryptophan residues in LmPEX7 were mostly unquenched and could be localized in regions inaccessible to the quencher.

**Figure 19. Fluorescence quenching.** 20 μM LmPEX7 in TBS was titrated with increasing concentrations of potassium iodide (KI) (black line) or acrylamide (gray line) (80 mM – 800 mM) and LmPEX7 intrinsic fluorescence was measured at 295 nm excitation wavelength and the emission collected at 300-450 nm. Baseline spectra were subtracted from the sample measurements and results were analyzed based on the Stern-Volmer equation. The Stern-Volmer constant ($K_{SV}$) for collisional quenching was determined by plotting the quencher concentration against the ratio of the fluorescence intensity in the absence ($F_0$) and presence of quencher ($F$) at 366 $\lambda_{max}$. 

148
Discussion

The PTS1 pathway in trypanosomatids and its relation to the PTS2 pathway in glycosomal protein has not yet been fully elucidated. The cloning and characterization of LmPEX7 in *L. donovani* suggests that this peroxin functions as a shuttling receptor and that the interaction between LmPEX7 and LdPEX5 plays a role in trafficking PTS2 proteins into the glycosomal matrix [29]. Although recombinant LmPEX7 which co-purified with GroEL functioned similarly as the native protein, it was important to show that this receptor is biologically active in the absence of the chaperonin. Whether a chaperone is important *in vivo* for the import of PTS2 proteins into the glycosome is unclear. However, studies with recombinant LmPEX7 revealed that the chemical chaperone glycerol was important in stabilizing this protein. Purification of LmPEX7 under denaturing conditions and refolding on the column yielded a soluble form of the protein that displayed similar functional characteristics.

A number of biophysical techniques were employed to examine the LmPEX7 structure. This receptor contains five conserved WD40 motifs, denoted by the sequence X$_{6-94}$-[GH-X$_{23-41}$-WD], which are postulated to form a β-propeller fold similar to the structure of the β-subunit of the trimeric G protein [46-48]. A WD40 motif forms a four-stranded β-propeller fold with the first three strands forming one propeller blade while the last strand forms part of the next blade. Members of the WD40 family are known to form homo- and hetero-oligomeric structures and this oligomerization is mediated by either the N- and C-terminal extensions found in these proteins [48]. The interior channel or pore of the propeller structure is considered to coordinate binding to other ligands [48-50]. In mammals, PEX7 has been postulated to form a similar β-propeller structure [47]. In this study, LmPEX7 formed tetrameric and dimeric structures based on cross-linking analyses and gel permeation chromatography with LmPEX7 forming mainly a tetramer upon binding a PTS2 ligand. In contrast, the mammalian PEX7 is proposed to be either a dimer or monomer [42, 43].
However, LmPEX7 consistently formed tetrameric structures even in the presence of LdPEX5, LdPEX14 or its PTS2 ligand as demonstrated by glycerol density gradient centrifugation which suggests that the tetrameric form of LmPEX7 is the more stable quaternary structure. The oligomeric structure of LmPEX7 is not surprising as LdPEX5 and LdPEX14 are known to form oligomers in solution with the latter forming structures with a molecular mass of ~800 kDa [32].

LmPEX7 was found to bind LdPEX5 at nanomolar binding affinity which was modulated by the PTS1 and PTS2 ligands. Interestingly, LdPEX5 increased the LmPEX7 affinity for PTS2 suggesting that LdPEX5 plays a role in stabilizing the LmPEX7-PTS2 complex during protein trafficking in the cytosol or at the glycosomal membrane prior to or during protein translocation. This contention is further strengthened by genetic analysis in T. brucei showing that the deletion of TbPEX5 resulted in the mistargeting of PTS2 proteins [35]. Similarly, PEX5 or PEX18 and PEX21 have been implicated in PTS2 peroxisomal import in mammalian and yeast peroxisomes [33, 51].

In contrast, the LmPEX7-LdPEX5 binding affinity substantially decreased in the presence of the PTS1 ligand LdXPRT. This leads to the notion that binding of an empty LdPEX5 receptor to LmPEX7 is required for the formation of the LmPEX7-PTS2 complex. The suggestion that the decrease in the LdPEX5-LmPEX7 binding affinity may be attributed to a steric phenomena generated by binding LdXPRT is unlikely since LdPEX5 is a bi-domain protein with the PTS1 binding site located between residues 300-625 while the LmPEX7 binding site is located within the N-terminal domain between residues 111-148 [27, 29].

Previous studies showed that the LdPEX5-LdPEX14 interaction decreases the LdPEX5-PTS1 binding affinity [32]. Similarly, the LmPEX7-PTS2 interaction was reduced upon binding LdPEX14 supporting the conjecture that LdPEX14 serves as a trigger for the dissociation of the receptor-cargo complexes required to release the cargo protein into the glycosomal matrix [32]. Although previous studies showed that LmPEX7 bound LdPEX14 in pull-down assays, the binding affinity between these two proteins is
relatively weak suggesting that the docking of LmPEX7-PTS2 on the membrane-associate LdPEX14 could be mediated by LdPEX5. Similarly, studies in mammalian peroxisomes showed that the long isoform of PEX5 (PEX5L) mediates the docking of PEX7 to the peroxisomal membrane [33].

These changes in binding affinities were associated with conformational changes that altered digestion patterns obtained by trypsin proteolysis. Significant degradation of LdPEX5 was observed when this receptor was loaded with PTS1 and complexed with LmPEX7 or LdPEX14 while the proteolysis-resistant LmPEX7 became more susceptible to degradation in the presence of either the empty or PTS1-loaded LdPEX5. Similarly, increased proteolysis of LdPEX14 was seen in the presence of the cargo-loaded receptors suggesting that these components undergo conformational changes upon complex formation which correspond to changes in binding affinities and tryptic digestion profiles.

The conformational changes in LmPEX7 induced by the presence of PTS2 and LdPEX5 were congruent with changes in the intrinsic fluorescence and surface hydrophobicity of LmPEX7. The increased fluorescence intensity of LmPEX7 and decreased surface hydrophobicity of LmPEX7 in the presence of both ldpx5 1-148 and ldald 1-77 that resulted in reduced ANS fluorescence are further evidence that this receptor underwent conformational change. The apparent high surface hydrophobicity of LmPEX7 alone explains why this protein is soluble in buffer containing glycerol.

The complex emission wavelength of LmPEX7 suggests that the tryptophan residues are located in polar and non-polar environments. The red-shifted emission wavelength ($\lambda_{max}$ 366 nm) of LmPEX7 is due to the aspartate residues adjacent to the tryptophans in the WD40 motifs. It has been shown that the electrostatic interactions between a charged residue near the pyrrole ring of tryptophan will shift the $\lambda_{max}$ to longer wavelengths [52]. The Stern-Volmer analysis of the quenching experiments on LmPEX7 suggests that the tryptophan residues in LmPEX7 were not readily quenched by KI or acrylamide indicating that the tryptophans are buried and less accessible to solvent or quencher.
However, the definitive localization of these tryptophan residues in the LmPEX7 structure will require crystallization of this protein.

The results of this study provided an insight into the interaction dynamics between LmPEX7 and other components of glycosomal protein import. The experiments determined the biophysical characteristics of LmPEX7 and highlighted the role of LdPEX5 in PTS2 protein import. The conformational changes in LmPEX7 induced by LdPEX5 could trigger LmPEX7 to assume an import-competent structure that results in the binding of PTS2 and subsequent docking on the glycosomal membrane. The modulation of the LmPEX7-LdPEX5 binding affinities by the PTS ligands denotes that the two import pathways require a coordinated series of interactions to translocate the PTS cargo proteins into the glycosome.
Chapter 4 References


Connecting Statement (Chapter 5)

The following chapter examined how LmPEX7 transports PTS2 proteins into the glycosome by mapping the domains important for binding the PTS2 ligand, LdPEX5, and LdPEX14, and for targeting to the glycosome to deliver its cargo protein. This study complemented the results in Chapter 4 to further elucidate the mechanism of glycosomal PTS2 sorting.
Chapter 5. Mapping the domains responsible for biological activity in the *Leishmania* glycosomal PTS2 receptor PEX7

Ana Victoria C. Pilar, Line Dufresne, Perunthottathu K. Umasankar, and Armando Jardim*

*Manuscript in preparation for submission

Abstract

The *Leishmania* PEX7 (LmPEX7) is a receptor required for the import of proteins containing a peroxisomal targeting sequence type 2 (PTS2) into the glycosome. LmPEX7 contains conserved WD40 motifs and bioinformatic analysis of LmPEX7 predicted a structure similar to the β-propeller fold of the β subunit of the trimeric G-protein, a model protein of the WD40 family. The model structure contained N- and C- terminal extensions and a central cavity that could function as protein interaction sites. Sequence alignment of the N- and C- terminal extensions found in trypanosomatid PEX7 showed low sequence similarity with yeast and mammalian homologs. Since the translocation of the LmPEX7 and its PTS2 ligand into the glycosomal matrix depends on an interaction with the PTS1 receptor LdPEX5 and the docking protein LdPEX14, a deletion mutagenesis analysis of LmPEX7 was performed to identify the motifs required for PTS2, LdPEX5, and LdPEX14 binding and to test whether the N- and C- terminal extensions function in ligand binding or targeting to the glycosome. These studies showed that the N-terminal portion of LmPEX7 is essential for binding LdPEX5 and LdPEX14. However, PTS2 binding actively requires the full-length protein LmPEX7. To
determine the specific domains required for LmPEX7 targeting to the glycosome, full-length and mutant LmPEX7 fragments containing the first 71 amino acid residues at the N-terminus or the C-terminal portion (aa 223-323) were expressed as GFP fusion proteins in *L. donovani* promastigotes. Expression of GFP-tagged full-length LmPEX7 in *Leishmania* confirmed that it functions similarly to the native LdPEX7. However, analysis of the subcellular localization of the GFP-tagged mutant LmPEX7 expressed in promastigotes is inconclusive as these mutants were unstable. Moreover, these mutants could not bind LdPEX5 and a PTS2 ligand Ldald 1-77 suggesting that the targeting of LmPEX7 requires a functional and full-length receptor. The development of a fluorescent live cell imaging or an inducible protein expression system in *L. donovani* should be able to address the targeting mechanism of LmPEX7.
Introduction

*Leishmania* and *Trypanosoma* are medically important parasites that cause a spectrum of devastating diseases such as visceral leishmaniasis and African sleeping sickness. These organisms also represent a unique model system for molecular and evolutionary biology studies because they diverged early from the main eukaryotic lineage and retained novel and distinct features that include the possession of a kinetoplast, a unique organelle called a glycosome, extensive RNA editing, and genes occurring in polycistronic clusters [1-4]. The glycosome is an important chemotherapeutic target that is evolutionarily related to the peroxisomes in other eukaryotes [5, 6]. Glycolysis is compartmentalized in the glycosomes along with other important metabolic pathways such as β-oxidation of fatty acids, ether lipid biosynthesis, and purine salvage [7, 8]. Interference with the proper functioning of these organelles is detrimental to the bloodstream form of *T. brucei* which is dependent on glycolysis for energy production [9-11].

Compartmentation of various processes in the glycosome means that an import mechanism is utilized to target proteins and metabolites into the glycosomal matrix as these organelles do not contain any DNA- or protein-synthesizing machinery unlike chloroplasts and mitochondria [12]. Proteins to be imported into the glycosome contain topogenic signals called peroxisomal targeting sequence (PTS) either at the C-terminus called PTS1 and is denoted by the archetype sequence –serine-lysine-leucine (SKL) or at the N-terminus (PTS2) characterized by the nonapeptide sequence R/K-L/V/I-X₅-Q/H-L/A [13, 14]. PTS proteins are synthesized on free cytosolic ribosomes and posttranslationally imported into the glycosome by binding to the receptors peroxin 5 (PEX5) and peroxin 7 (PEX7) which recognize the PTS1 and PTS2 signals, respectively [15-18]. Several models have been proposed for the import of proteins into the peroxisome and glycosome. The main steps in these models involve the formation of PEX5-PTS1 and PEX7-PTS2 complexes, docking of the receptor-cargo complex on the glycosomal or peroxisomal membrane through PEX14 or PEX13, translocation of the PTS proteins into the matrix, and shuttling of the receptors back into the cytosol [19, 20].
In the extended shuttle model proposed in yeast and mammals, the receptor-cargo complex is translocated into the matrix via the “importomer complex” which consists of the docking complex PEX14, PEX13, PEX17, and the RING complex formed by PEX2, PEX10, and PEX12 [21, 22]. Upon translocation across the membrane, the PTS protein is released into the matrix and the receptor is recycled back into the cytosol by interacting with PEX8 and the RING complex [22, 23]. In the transient pore model, PEX5 is proposed to assemble a pore consisting of oligomeric units that inserts into the peroxisome membrane through which PTS proteins are translocated [19, 24]. The pore is disassembled in an ATP-dependent manner via the AAA+ proteins, PEX1 and PEX6, and by the RING complex which is proposed to ubiquitinate PEX5 to facilitate recycling to the cytosol [25-29]. Although the steps for PTS2 protein import have not been fully elucidated, it is assumed that a similar process takes place in this pathway [19, 22].

The details of the mechanism of protein import in glycosomes are not well-understood but the decoding of several kinetoplastid genomes has facilitated the identification of several key proteins required for this process. More than 30 peroxins have been identified to participate in peroxisome biogenesis, however, only 10-15 homologs have been identified in glycosomes and the function of most of these proteins has yet to be determined [6]. In Leishmania, the docking protein LdPEX14 and the receptors PEX5 (LdPEX5), and PEX7 (LmPEX7) have low sequence similarity to their peroxisomal homologues while PEX13 has yet to be identified in Leishmania sp. [30-32]. The recent identification of the RING proteins PEX2, PEX10, and PEX12 in T. brucei and Leishmania suggests that a similar importomer complex could be present in trypanosomatids [33]. The interactions of the receptors and LdPEX14 are mediated by novel binding motifs not found in other eukaryotic peroxins [30-32, 34]. After receptor-cargo complex formation, the docking of LdPEX5-PTS1 on LdPEX14 results in reduced binding affinity of LdPEX5 to PTS1 suggesting that the interaction of LdPEX5 and LdPEX14 could serve to release the cargo protein into the matrix [35]. A similar event could take place during PTS2 import as ELISA-based interaction assays showed that the presence of LdPEX14 reduces the binding affinity of the LmPEX7-PTS2 complex (Pilar, et al. in prep).
Recent studies on the *L. donovani* PEX7 show that this protein is located in the cytosol and glycosomal matrix suggesting that it functions as a shuttling receptor [32]. Binding assays demonstrated that the LmPEX7 also interacts with LdPEX5 but whether this interaction is required for protein import is unknown. The importance of these receptors in glycosomal functioning can be gleaned from RNA interference studies in *T. brucei* showing that reducing the levels of TbPEX5 and TbPEX7 caused improper targeting of glycosomal proteins [11]. Genetic analysis of glycosomal protein interactions has been impeded by the difficulties in obtaining Δldpex5 and Δldpex14 null mutants in *Leishmania* and the lack of the RNAi mechanism in *L. donovani* [30, 36].

To gain more insight into the role of the LmPEX7 in PTS2 protein import in *Leishmania*, we determined the domains on LmPEX7 responsible for its biological activity. This investigation elucidates the targeting of LmPEX7 to the glycosome and the molecular features of LmPEX7 that are important for interacting with the other components of the glycosomal import machinery.
Materials and methods

Materials and reagents

Restriction enzymes and other DNA-modifying reagents were procured from either Invitrogen Life Technologies (Grand Island, NY) or New England Biolabs (NEB, Beverly, MA). Horseradish peroxidase-conjugated IgG secondary antibodies were obtained from GE Healthcare Life Sciences (Piscataway, NJ) and Cedarlane Biolabs (Burlington, ON). FITC or Cy3-conjugated IgG secondary antibodies were from Jackson Immunoresearch (Westgrove, PA). Mini-EDTA free protease inhibitor cocktail was from Roche Molecular Biochemicals (Indianapolis, IN). Ni^{2+}-nitrilotriacetic acid agarose beads were obtained from QIAGEN (Mississauga, ON), chitin beads were purchased from New England Biolabs (NEB) (Ipswich, MA), and S-protein agarose beads were procured from Novagen (Madison, WI). All other reagents were of research grade quality commercially available.

*L. donovani* strain DI700 promastigotes were cultured at 26 °C in Dulbecco’s modified Eagle-Leishmania (DME-L) with penicillin/streptomycin, 5.0 mg/l hemin, 100 μM hypoxanthine, and 10% heat-inactivated fetal bovine serum.

*LmPEX7* modeling and sequence alignments

A three-dimensional computational model of LmPEX7 was generated using the I-TASSER server [37]. Image manipulations and presentation were performed using the SwissPdB viewer [38]. Sequence alignments were done using the software Clustal W 2.0.8 [39].
**LmPEX7 constructs**

*pET30(b)-lmpex7 1-223-His₆ (NT)* containing the first 223 amino acids of LmPEX7 was generated by digesting *pET30(b)-LmPEX7-His₆* with *Nde*I and *Sal*I and ligating the fragment into the corresponding sites in the plasmid *pET30(b)+* (Novagen) using T4 DNA ligase (Invitrogen). The *pBAce-lmpex7 223-373 (CT)* construct encoding amino acids 223-373 was generated by digesting *pET30(b)-LmPEX7-His₆* with *Sst*I, polishing the ends with T4 DNA polymerase (Invitrogen), and digesting with *Sal*I. This fragment was cloned into the *pBACE* vector [40] which was previously digested with *SsrI*, blunted with T4 DNA polymerase (Invitrogen) then digested with *Sal*I, and treated with calf intestinal phosphatase (NEB). To generate *pBAce-PT5-lmpex7 223-373-His₆*, the phage T5 promoter and lac operator from the vector *pQE81L* was amplified by PCR using the primers 5’-*CGGGGTACCAATGATTTTTTAATT-3’ (*Kpn*I restriction site underlined), 5’-*CATGCCATGGTTAATTTCTCCT-3’ (*Nco*I restriction site underlined), and *Pfu* DNA polymerase (Invitrogen) at 95 °C initial denaturation, 29 cycles of 95 °C denaturation for 45s, 56 °C annealing for 30s, and 68 °C extension for 30s with a 10 min final extension at 68 °C. The PCR product was digested with *Kpn*I and *Nco*I and ligated into the corresponding restriction sites of *pBAce-lmpex7 223-373-His₆*.

LmPEX7 fused to the N-terminus of the green fluorescent protein (GFP) was created by PCR amplification of the LmPEX7 open reading frame (ORF) using *pET30(b)-LmPEX7-His₆* as template and the primers 5’-*GTCGGATCCATGCCGCTTCCCTCCAGATGCACCC-3’ and 5’-*CCGGGGATCCCGAGGGGAACGCCTGGAGACGACG-3’ (*BamHI* restriction sites underlined), and *Pfu* DNA polymerase using the same PCR conditions described above. The PCR product was digested with *BamHI* and ligated into the corresponding sites in the vector *pXG-GFP* [41] to create *pXG-LmPEX7-GFP*. To generate *pXG-lmpex7 1-71-GFP*, a GFP fusion protein containing the first 71 amino acids of LmPEX7, the vector *pXG-LmPEX7-GFP* was digested with *SmaI* and *EcoRV*. The LmPEX7 ORF was digested with *MluI* and the ~200 bp fragment was treated with large Klenow fragment DNA polymerase I (NEB) and ligated into the *SmaI* site of *pXG-GFP*. To create *pNUS-lmpex7*
223-373-GFPcN, a C-terminal fragment encoding amino acids 223-373 of LmPEX7 fused to GFP, the fragment was amplified by PCR using pBAce-PT5-lmpex7 223-373-His$_6$ as a template, the primers 5’-GGATTCCATATG GTCGACCGCACCGTCC-3’ (NdeI restriction site underlined), 5’-GGGGTACCAAGCTTTCCGAGGGGAACG-3’ (KpnI site restriction site underlined), and Pfx DNA polymerase using the PCR conditions described above. The PCR product was digested with NdeI and KpnI and ligated into the corresponding restriction sites in the vector pNUS-GFP-cN [42]. The DNA sequences of all constructs were verified by automated DNA sequencing analysis.

Expression and purification of recombinant proteins

The expression and purification of LmPEX7-His$_6$, His$_6$/S-LdPEX14, and ldald 1-77-His$_6$, a fragment containing the first 77 amino acids of the L. donovani fructose-1,6-bisphosphate aldolase were performed as previously described using Ni$^{2+}$-nitritoltriacetic acid (NTA) affinity column chromatography (Qiagen) [31, 32]. LdPEX5 protein was prepared as previously reported using a chitin affinity column (Novagen) [30]. ldpex5NT, an LdPEX5 fragment containing residues 1-391, was purified as described previously [30].

E. coli ER2566 transformed with the plasmids pET30(b)-lmpex7 1-223-His$_6$, and pBAce-PT5-lmpex7 223-373-His$_6$ were grown to an OD$_{600}$ 0.7 at 37 °C in Luria Broth with 100 µg/ml kanamycin or ampicillin. Protein expression was induced for 4 h at 25 °C with 0.7 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested and the pellets resuspended in 20 mM Tris-HCl pH 8.0 with an EDTA-protease inhibitor cocktail (Roche) and lysed with two passes through a French press. Lysates were clarified and loaded onto a Ni$^{2+}$-NTA column. Proteins were eluted with an imidazole gradient (10-320 mM) in 40 mM Tris-HCl pH 8.0, 500 mM NaCl (TBS) following manufacturer’s instructions (Qiagen) and concentrated using a 5,000 NMWL centrifugal device (Millipore).
Leishmania transfection

Mid-log phase *L. donovani* promastigotes were harvested (2 x 10^8 cells), washed with PBS and electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl₂, 5 mM KCl₂, 0.7 mM Na₂HPO₄, and 6 mM glucose), and diluted to a final concentration of 1 x 10^8 cells. For low-voltage transfection, 10 μg of purified pXG-LmPEX7-GFP, pXG-lmpex7 1-223-GFP, or pNUS-lmpex7 223-373-GFPcN was mixed with 400 μl of cells (4 x 10^7) in a 2 mm-gap electroporation cuvette (Fisher Scientific) and incubated on ice for 10 min. Electroporation was performed by pulsing cells once at 450 V and 500 μF then cells were transferred to DME-L media and grown at 26 °C for 24-48 h [43]. Neomycin-resistant transfectants were selected by the addition of 20 μg/ml G418 for LmPEX7-GFP transfectants and 200 μg/ml G418 for both lmpex7 1-71-GFP and lmpex7 CT-GFP-expressing transfectants.

Protein binding assays

Covalent coupling of LmPEX7-His₆, LdPEX5, ldpex5NT, and ldald 1-77-His₆ to Affigel-10 agarose beads (BioRad) and binding of His₆/S-LdPEX14 to S-beads were performed as described previously [32]. Pull-down assays were done by mixing 50 μl packed beads with 20 μg purified LmPEX7-His₆, lmpex7 1-223-His₆ (lmpex7NT-His₆) or lmpex7 223-373-His₆ (lmpex7CT-His₆) and incubating for 30 min with occasional mixing at 20 °C. The beads were washed 3 x 1% Triton X-100 in TBS (TBST) and 3 x TBS. Bound proteins were analyzed by SDS-PAGE and Western blot. For pull-down assays to characterize LmPEX7-GFP transgenic *L. donovani* promastigotes, 1 x 10^8 mid-log phase promastigotes were harvested, washed twice with PBS, and lysed with 1% Triton X-100 in TBS containing a protease inhibitor cocktail (Roche). The lysates were centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant incubated with 100 μl packed affinity beads (LmPEX7-His₆, ldpex5NT) for 16 h at 4 °C. The beads were washed and bound proteins were analyzed by Western blot. For control experiments, bovine serum albumin
(BSA) coupled to Affigel-10 beads were incubated with the recombinant proteins or promastigote cell extracts.

**Confocal microscopy analysis**

Mid-log phase wild-type (WT) and transfected promastigotes were attached to poly-L-lysine-coated coverslips, fixed for 10 min with 4% formaldehyde in PBS, blocked, and permeabilized with 0.25% Triton X-100, 50 mM Tris-HCl pH 7.5, 2% BSA in PBS for 20 min. Cells were incubated for 1 h at 20 °C with the primary guinea pig anti-LmPEX7 antibody (1:100) and rabbit anti-GFP (1:50) diluted in PBS/2% BSA. For co-localization, parasites were co-stained with the antibodies rabbit anti-hexokinase (1:500), guinea pig anti-inosine monophosphate dehydrogenase (LdIMPDH) (1:200), rabbit anti-xanthine phosphoribosyltransferase (LdXPRT) (1:100), and rabbit anti-adenine phosphoribosyltransferase (LdAPRT) (1:100). Cells were then stained with donkey anti-guinea pig FITC-conjugated (1:1,000), goat anti-guinea pig Cy3-conjugated (1:1,000), and mouse anti-rabbit Cy3-conjugated secondary antibodies (1:1,200) (Jackson Immunoresearch).

**Subcellular fractionation**

Subcellular fractionation of wild type and transfected promastigotes on sucrose density gradient centrifugation and protease protection assays were performed as detailed previously [32, 44]. Briefly, mid-log phase promastigotes (2 x 10^8 cells/ml) were suspended in hypotonic buffer (5 mM HEPES pH 7.4, 2 mM EGTA, 2 mM DTT, protease inhibitor cocktail) and lysed by passing the cells 20 times through a 27-gauge needle. The lysates were made isotonic by adding 4X assay buffer (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 °C to remove intact cells and nuclei. The supernatant was overlaid on a 20-70% linear sucrose gradient and centrifuged at 218,000
x g for 16 h at 4 °C. The gradients were fractionated by collecting aliquots from the bottom of the tube and proteins precipitated using chloroform-methanol.

Protease protection assays were performed by obtaining *L. donovani* post-nuclear lysates as described above. The lysates were centrifuged at 40,000 x g for 40 min at 4 °C to obtain a light mitochondrial pellet. The pellet was resuspended in 1X assay buffer and treated with 150 μg/ml protease K (Invitrogen) for 30 min at 0 °C in the presence or absence of 1% Triton X-100. Reactions were terminated by trichloroacetic acid (TCA) precipitation. All samples were resolved in SDS-PAGE and analyzed by Western blot.

*Western blot analysis*

Proteins resolved on a 10% SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 3% skim milk, 0.05% Tween-20 in TBS. Guinea pig anti-LmPEX7 (1:10,000), rabbit anti-LdPEX5 (1:5,000), rabbit anti-LdPEX14 (1:10,000), rabbit anti-LdXPRT (1:2,000), guinea pig anti-LdIMPDH (1:2,000), and rabbit anti-β-tubulin (1:10,000) were used as primary antibodies while donkey anti-guinea HRP-conjugated and mouse anti-rabbit HRP-conjugated secondary antibodies were used (Cedarlane and GE Healthcare). Descriptions of primary antibodies used in this study were presented elsewhere (Pilar, et al. *in prep*).
Results

LmPEX7 modeling

Proteins belonging to the WD40 family are involved in diverse cellular functions such as signal transduction, cell division, and transcriptional regulation [45]. The WD40 motif consists of four to 16 repeats with a consensus sequence denoted by $X_{6-94} \cdot [GHI-3x41-\text{WD}]$ [46]. The crystal structure of the model WD40 protein, β-subunit of the trimeric G-protein, shows a three-dimensional seven-bladed β-propeller structure. The WD40 repeat folds into four anti-parallel β-strands with the first three β-strands forming one propeller blade while the fourth one is found in the next blade. The N-terminal portion of the β-propeller overlaps with the seventh blade to close the ring [45-47].

The predicted three-dimensional structure of LmPEX7 generated using I-TASSER showed a similar β-propeller structure (Fig. 20A & B) with the conserved WD40 motifs forming β-strands in the seven propeller blades. The eight conserved cysteine residues (highlighted in green) were found within the WD motifs and propeller blades. Interesting features of this structure are the flexible N- and C-terminal extensions consisting of amino acid residues 1-14 and 363-373 (Fig. 20B) which adopt a coiled conformation. The cavity or tunnel at the center of the protein is lined by hydrophobic amino acid residues and have a diameter of ~10-12 angstroms. The outer surfaces of the β-propeller contain mostly hydrophobic residues and seven of the ten tryptophan residues (highlighted in blue) are buried inside the propeller folds as part of the WD40 repeats (highlighted in red).

Sequence alignments of the first 71 amino acids of LmPEX7 and the C-terminal portion spanning residues 223-373 showed limited sequence identity with corresponding PEX7 regions in *S. cerevisiae* and *H. sapiens* (Fig. 21A & B). The N- and C-terminal extensions of LmPEX7 had 94-96% identity with the *L. infantum* (*L. donovani* species complex) homolog and 61-64% with the *T. brucei* PEX7 but only 16-21% identity is
shared with the yeast and human PEX7 suggesting that the N- and C-terminal regions are unique to trypanosomatids. The N-terminal extension contains the consensus sequence PGX₂GX₆PX₁₀F/YGX₂GXGX₉G while the C-terminus contains poly-proline repeats which are lacking in the yeast and human PEX7 [32].

**Functional characterization of LmPEX7**

To determine the portion of LmPEX7 required for protein-protein interactions, lmpex7NT-His₆ (LmPEX7NT), an LmPEX7 mutant construct which contains the first 223 amino acids and lmpex7CT-His₆ (LmPEX7CT), a fragment spanning residues 223-373, were overexpressed in *E. coli* and migrated as ~25 kDa proteins. *In vitro* pull-down assays using LdPEX5 and His₆/S-LdPEX14 affinity beads showed that these resins precipitated the full-length LmPEX7 and to a lesser extent, lmpex7NT (Fig. 22A lanes 1 & 2). As a positive control, His₆/S-LdPEX14 affinity beads were also incubated with ldpex5NT, an N-terminal fragment of LdPEX5 containing amino acids 1-323, which contains the LdPEX14 binding domain (Fig. 22A lane 4) [34]. Pull-down assays using ldald 1-77 beads (Fig. 22B) precipitated the full-length LmPEX7 (Fig. 22B lane 1) but not lmpex7NT and lmpex7CT (Fig. 22B lanes 2 & 3) suggesting that the N-terminal portion of LmPEX7 plays a role in binding LdPEX5 and LdPEX14 but an intact LmPEX7 is needed for PTS2 binding. The supernatants from the pull-down assays were also analyzed by Western blot to show the presence of unbound proteins (Fig. 22B lanes 5-7). The bands observed in the blots were specific for LmPEX7 as affinity beads incubated with buffer alone did not show any bands (Fig. 22A lanes 5 & 9, Fig. 22B lane 4). Binding assays using either LdPEX5 or His₆/S-LdPEX14 affinity beads incubated with a mixture of the N- and C-terminal fragments showed that only the N-terminal portion was functional and that the two mutant proteins could not dimerize to form a whole protein (*data not shown*).
Figure 20. Predicted structure of LmPEX7. (A) A computer model of the structure of LmPEX7 constructed using the I-TASSER server. Image manipulations and presentation were done using the SwisPdB software. The β-propeller structure of LmPEX7 contains seven blades consisting of β-strands formed by the conserved WD40 motifs (white ribbons). The conserved cysteine residues are emphasized in green. Three tryptophan residues that are possibly exposed are highlighted in red while tryptophan residues buried within the structure and forming the β-strands are colored blue. The N-terminal extension, consisting of amino acid residues 1-14, is colored yellow while the C-terminal region spanning amino acids 363-373 is highlighted in red. The first amino acid methionine and terminal residue arginine are represented as MET1 and OXT373, respectively. (B) Side view of the propeller structure of LmPEX7 showing the free N- and C-terminal regions (yellow & red).
Figure 21. Sequence alignments of N- and C- terminal regions of LmPEX7. Sequence alignment of the N- terminal portion of LmPEX7 spanning residues 1-71 (A) and the C-terminal region from amino acids 223-373 (B) with PEX7 homologs in L. infantum, T. brucei, S. cerevisiae, and H. sapiens. Asterisks denote conserved identical residues, colons denote conserved amino acid substitutions, and periods represent semi-conserved substitutions. The conserved WD motifs are boxed, the consensus sequence found in the N-terminus is highlighted in bold while the poly-proline repeats at the C-terminus are underlined. Alignments were performed using Clustal W 2.0.8.
Expression of LmPEX7-GFP fusion proteins

To determine the part of LmPEX7 required for glycosome targeting, full-length LmPEX7, lmpex7 1-71-GFP, and lmpex7 223-373-GFP (lmpex7 CT-GFP) were expressed in L. donovani promastigotes as GFP-fusion proteins to determine the subcellular distribution. Western blot analysis of wild type and transgenic parasites using anti-LmPEX7 and anti-GFP revealed that protein expression levels of LmPEX7-GFP, normalized to β-tubulin, did not alter the levels of native LdPEX7 (Fig. 23A). However, Western blot analysis of transgenic parasites expressing lmpex7 1-71-GFP (Fig. 23B lane 1) showed a lower expression level of the fusion protein compared to lmpex7 CT-GFP (Fig. 23B lane 2). Control parasites transfected with the pXG-GFP plasmid containing a mutation at the start codon of GFP [41] expressed only the native LdPEX7 which had similar levels of expression as the native LdPEX7 expressed in lmpex7 CT-GFP-transfected parasites (Fig. 23C). The wild type cells had similar growth rates and morphology as the transgenic parasites grown at the same drug concentration.

Immunofluorescence of LmPEX7-GFP fusion proteins

Confocal microscopy analysis of the LmPEX7-GFP transfected parasites showed punctate GFP staining that co-localized with the glycosomal marker hexokinase (Fig. 24A-D) [48]. In contrast, transgenic parasites expressing lmpex7 1-71-GFP (Fig. 24E-H) and lmpex7 CT-GFP (Fig. 24I-L) showed predominantly cytosolic GFP staining. Parasites transfected with the empty vector pXG-GFP did not have GFP fluorescence and showed punctate staining pattern for hexokinase denoting glycosomal localization (Fig. 24M-P)
Figure 22. Functional characterization of LmPEX7. LdPEX5, His\textsubscript{6}/S-LdPEX14, and ldald 1-77-His\textsubscript{6} were coupled to affinity beads (50 μl) and incubated with 20 μg of LmPEX7-His\textsubscript{6}, Impex7NT-His\textsubscript{6}, and Impex7CT-His\textsubscript{6} in 40 mM Tris-HCl pH 8.0, 500 mM NaCl (TBS). After washing the beads under stringent conditions, bound proteins were resolved by SDS-PAGE and analyzed by Western blot using guinea pig anti-LmPEX7 (1:10,000) and rabbit anti-LdPEX5 (1:10,000). (A) His\textsubscript{6}/S-LdPEX14 S-beads (lanes 1-5) and LdPEX5 Affigel beads (lanes 6-9) mixed with LmPEX7-His\textsubscript{6} (lanes 1 & 6), Impex7NT-His\textsubscript{6} (lanes 2 & 7), Impex7CT-His\textsubscript{6} (lanes 3 & 8), ldpe5NT (lane 4), and buffer alone (lanes 5 & 9). (B) PTS2-containing protein ldald 1-77-His\textsubscript{6} agarose beads incubated with LmPEX7-His\textsubscript{6} (lane 1), Impex7NT-His\textsubscript{6} (lane 2), Impex7CT-His\textsubscript{6} (lane 3), and buffer aloe (lane 4). Unbound supernatant from the binding assays were also analyzed by Western blot: lane 5 – LmPEX7-His\textsubscript{6}, lane 6- Impex7NT-His\textsubscript{6}, and lane 7 – Impex7CT-His\textsubscript{6}. 

<table>
<thead>
<tr>
<th>Beads</th>
<th>His\textsubscript{6}/S-LdPEX14</th>
<th>LdPEX5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1, 2, 3, 4, 5</td>
<td>6, 7, 8, 9</td>
</tr>
<tr>
<td>kDa</td>
<td>50, 37, 25</td>
<td></td>
</tr>
<tr>
<td>Ldpe5NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LmPEX7-His\textsubscript{6}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impex7NT-His\textsubscript{6}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ldald 1-77-His\textsubscript{6} beads</th>
<th>Unbound supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3, 4</td>
<td>5, 6, 7</td>
</tr>
<tr>
<td>kDa</td>
<td>LmPEX7-His\textsubscript{6}</td>
</tr>
<tr>
<td></td>
<td>Impex7 CT-His\textsubscript{6}</td>
</tr>
</tbody>
</table>
Figure 23. Expression of GFP-tagged LmPEX7 in *L. donovani* promastigotes. Mid-log phase promastigotes were transfected with *pXG-LmPEX7-GFP, pXG-Impex7 1-71-GFP, pNUS-Impex7 CT-GFP-cN, and pXG-GFP* and the parasites were grown in DME-L with 20-200 µg/ml G418. To determine the expression of the GFP-tagged proteins, cells were lysed with 1% Triton X-100 in TBS and the cell extracts were resolved in SDS-PAGE and analyzed by Western blot using guinea pig anti-LmPEX7 (1:2,000), rabbit anti-GFP (1:200), and rabbit anti-tubulin (1:10,000). (A) Cell extracts of wild type parasites (lane 1) and LmPEX7-GFP transfected cells (lane 2). (B) Cell extracts of Impex7 1-71-GFP (lane 1) and Impex7 CT-GFP (lane 2). (C) Cell extracts of Impex7 CT-GFP (lane 1) promastigotes and cells transfected with the empty vector pXG-GFP (lane 2).
Figure 24. Confocal microscopy analysis of GFP-tagged LmPEX7. Transfected *L. donovani* promastigotes were fixed with 4% paraformaldehyde in PBS, blocked, and permeabilized with 0.25% Triton X-100, 50 mM Tris-HCl pH 7.5 and 3% bovine serum albumin (BSA) in PBS. Panels A-D – LmPEX7-GFP transfectants co-stained with the glycosomal marker hexokinase using anti-hexokinase (1:500) and anti-rabbit Cy3 (1:1,200). Cells expressing Impex7 1-71-GFP (panels E-H) and Impex7 CT-GFP (panels I-L) were incubated with rabbit anti-GFP (1:50) and stained with mouse anti-rabbit FITC (1:1000). For glycosomal co-localization, cells were co-stained with anti-IMPDH (1:100) and Cy3-conjugated goat anti-guinea pig antibody (1:1,000). Panels M-P – cells transfected with *pXG-GFP* stained with anti-hexokinase (1:500) and Cy3-conjugated mouse anti-rabbit antibody (1:1,200). Panels C, G, K, O – merged images of stained cells to show localization. Panels D, H, L, P – phase contrast images of stained cells.
To further determine the localization of the LmPEX7-GFP fusion proteins, sucrose density gradient centrifugation and protease protection assays were performed. Native LdPEX7 localized both to the cytosolic and glycosomal fractions in the sucrose gradient and co-sedimented with LdPEX14 and the glycosomal enzyme IMPDH similar to previous studies (Fig. 25A) [32]. Western blot analysis of the subcellular fractionation of LmPEX7-GFP transgenic parasites using anti-GFP antibodies showed that LmPEX7-GFP was found predominantly in the glycosomal fractions with a small population localized to the cytosol (Fig. 25B). Glycosomal LmPEX7-GFP also co-localized with LdPEX14 and the glycosomal marker LdXPRT. The cytosolic marker LdAPRT was found only in the top portion of the gradient denoting cytosolic fractions (Fig. 25B). The presence of LdXPRT and LdPEX14 in the cytosolic fractions could be due to disruption of some glycosomes during cell lysis. In contrast to LmPEX7-GFP, Lmpex7 1-71-GFP and Lmpex7 CT-GFP were found mostly in the upper portion of the sucrose gradient denoting cytosolic fractions (Fig. 25D) suggesting that these fragments are not targeting to the glycosome and that the mutant proteins could be forming protein aggregates that migrated inner into the sucrose gradient.

To determine whether LmPEX7-GFP is found in the glycosomal matrix, protease protection assays were done in the presence or absence of Triton X-100 and the digests analyzed by Western blot using anti-GFP antisera. LmPEX7-GFP and the glycosomal marker LdIMPDH were resistant to proteolysis when the post-nuclear lysates were treated with protease K confirming that LmPEX7-GFP could target into the glycosomes similar to the native LdPEX7 and that adding a GFP tag on LmPEX7 does not affect its ability to enter glycosomes (Fig. 26). In contrast, LdPEX14 was easily degraded by protease K as it is situated on the outer surface of the glycosomal membrane as a peripheral membrane protein (Fig. 26 top most panel).
To further determine the localization of Impex7 1-71-GFP and Impex7 CT-GFP, protease protection assays were performed using the 40,000 x g pellet from postnuclear lysates of the transgenic promastigotes that contains mostly light mitochondria, lysosomes, and glycosomes. Western blot analysis showed that, similar to LmPEX7-GFP, Impex7 1-71-GFP and Impex7 CT-GFP were resistant to proteolysis (Fig. 26 lower most panel) and in the presence of detergent, all proteins were readily degraded by protease K (Fig. 26 lane 3).

**Binding assays of GFP-tagged LmPEX7 mutants**

To functionally characterize the LmPEX7-GFP fusion proteins, pull-down assays were performed using ldpex5NT, an LdPEX5 fragment containing the LmPEX7 binding domain, and ldald 1-77 affinity beads incubated with detergent cells extracts from the transfected parasites (Fig. 27A & B). The pull-down assays showed that both the native (Fig. 27A lane 1) and LmPEX7-GFP (Fig. 27A lane 2) proteins interacted with ldpex5NT and that a ternary complex could be observed consisting of *Leishmania* hexokinase-PEX7-PEX5-PEX14 as both LdPEX14 and the PTS2 protein hexokinase were also pulled-down by the beads. Lesser amounts of LdPEX14 and LdPEX7 were precipitated by ldpex5NT when incubated with wild type promastigote cell extracts (Fig. 27 lane 1) compared to that of LmPEX7-GFP transgenic parasites (Fig. 27 lane 2). This could be due to the combined amounts of LmPEX7-GFP and native LdPEX7 binding more ldpex5NT and LdPEX14.

Similarly, ldald 1-77 affinity beads precipitated both the native and GFP-tagged LmPEX7. Interestingly, the PTS2 enzyme hexokinase was also pulled-down by the ldald 1-77 beads which could be due to the former enzyme binding to oligomeric structures of LdPEX7. These results suggest that both LmPEX7-GFP and the native LdPEX7 have similar functions.
In contrast, binding assays using ldpex5NT affinity beads did not precipitate LmPEX7 1-71-GFP or LmPEX7 CT-GFP (Fig. 27B lanes 3 & 4) but the beads bound strongly to the native LdPEX7 (Fig. 27B lane 1) and LmPEX7-GFP (lane 2). As a control, Western blot analysis of the unbound supernatant from the assays was performed to show the presence of LmPEX7 1-71-GFP and LmPEX7 CT-GFP in the detergent cell extracts (Fig. 27B lane 9). Similar binding assays using ldald 1-77 affinity beads showed no interaction between the PTS2 ligand and the LmPEX7 mutant fragments (data not shown). This supports the results of binding assays using the recombinant LmPEX7 mutant fragments and suggests that mutating LmPEX7 affects its ability to interact with other components of the glycosomal import pathway.
Figure 25. Subcellular fractionation of GFP-tagged LmPEX7. (A-D) Wild-type and transgenic promastigote cell extracts were prepared by hypotonic lysis and post-nuclear supernatants were overlayed on top of a 20-70% sucrose gradient and fractionated at 218,000 x g for 16 h at 4°C. Fractions were precipitated with chloroform-methanol and analyzed by Western blot using guinea pig anti-LmPEX7 (1:2,000), rabbit anti-GFP (1:100) and for glycosomal localization, rabbit anti-LdPEX14 (1:10,000), rabbit anti-LdXPRT (1:2,000), and guinea pig anti-LdIMPDH primary antibodies were used. Rabbit anti-LdAPRT primary antibodies were used to denote cytosolic fractions. Panel A- wild type promastigotes; panel B- LmPEX7-GFP transfected cells; panel C- Impex7 1-71-GFP transfected cells; panel D – Impex7 CT-GFP cells. Lane 1 – 3,000 x g pellet (P) from crude lysates containing intact cells and cytosolic components.
Figure 26. **Protease protection assays.** Cell extracts of transfected promastigotes were prepared by hypotonic lysis and treated with 150 μg/ml proteinase K (prot K) in the presence or absence of Triton X-100 (TX) for 30 min on ice. Reactions were terminated by TCA precipitation and digests were analyzed by Western blot using rabbit anti-LdPEX14, guinea pig anti-LdIMPDH, guinea pig anti-LmPEX7, and rabbit anti-GFP.
**Figure 27. Binding assays of GFP-tagged LmPEX7.** 50 μl Affigel beads coupled to ldpx5NT (A) and ldald 1-77-His6 (B) were incubated with detergent cell extracts of wild type (lane 1) and LmPEX7-GFP (lane 2) transfected promastigotes overnight at 4°C. Beads were washed under stringent conditions with TBS and the bound proteins were analyzed by Western blot using guinea pig anti-LmPEX7 (1:2,000), rabbit anti-GFP (1:100), rabbit anti-hexokinase (1:10,000), and rabbit anti-LdPEX14 (1:10,000). (C) Pull-down assays using 50 μl ldpx5NT Affigel beads incubated with cell extracts of wild type promastigotes (WT, lane 1), LmPEX7-GFP (7GFP, lane 2), Impex7 1-71-GFP cells (1-71, lane 3), and Impex7 CT-GFP (CT, lane 4) transfected promastigotes. Lane 5, C-control affinity beads coupled to BSA and incubated with cell extracts of wild type promastigotes. Lane 6, U – supernatant from the binding assays showing the presence of unbound LmPEX7-GFP, Impex7 1-71-GFP, Impex7 CT-GFP, and native LdPEX7. Blots were probed with rabbit anti-GFP (1:200) and guinea pig anti-LmPEX7 (1:2,000).
Discussion

Elucidation of the mechanism of PTS2 protein import into the glycosomes in trypanosomatids is important as several PTS2 enzymes such as hexokinase and aldolase must be targeted to the glycosome for parasite viability and are considered drug targets [49, 50]. We previously characterized the *Leishmania* PEX7 and showed that this receptor bound PTS2 ligands and interacted with LdPEX5 and LdPEX14 [32]. The importance of the LmPEX7-LdPEX5 interaction in the PTS2 import pathway and in relation to the formation of the docking complex with LdPEX14 is currently being investigated (Pilar, et al. *in prep*). During the import of PTS2 proteins, LmPEX7 may function as a shuttling receptor as this protein was found in the cytosol and also accumulated inside the glycosomes. The apparent accumulation of LmPEX7 in the glycosome could be due to the faster translocation of the cargo-laden receptor into the glycosomal matrix than its relocation back to the cytosol [32]. How LmPEX7 crosses the glycosomal membrane to deliver its cargo or whether it goes through a translocon is unknown. The components of a translocon, aside from LdPEX14, have yet to be identified or characterized while the formation of a transient pore by PEX5 as proposed in peroxisomes [19] has yet to be proven in glycosomes.

In this study, we determined the domains on LmPEX7 that are important for protein-protein interactions and for targeting into the glycosomes. Using modeling software, the predicted structure of LmPEX7 showed a β-propeller structure similar to the WD40 family of proteins with free N- and C- terminal extensions. A β-propeller structure contains potential binding surfaces at the top, bottom, and along the circumference of the structure [46]. The tunnel in the middle of the β-propeller is postulated to play a role in coordinating interactions with other molecules or ligands [45, 51]. Some WD40 proteins contain N- and C- terminal extensions that adopt a helical structure and have been implicated in binding other ligands or WD40 proteins as was observed in the β-subunit of the trimeric G protein [46]. The various surfaces of the propeller blades made up of the WD40 repeats offer hydrophobic surfaces that could also be important platforms for
binding other proteins, thus illustrating the versatility of this tertiary structure [45]. Members of the WD40 family have been shown to form homo- or hetero-oligomers with other WD40 proteins [46]. Structural analysis of LmPEX7 has shown that it predominantly forms tetrameric structures (Pilar, et al. in prep). Whether LmPEX7 behaves similarly to other WD40 proteins in terms of ligand binding needs further investigation.

The crystal structures of the *T. brucei* and *L. mexicana* aldolase demonstrate that the PTS2 signal in this homotetrameric enzyme forms a dimer via hydrophobic residues [52] but whether the enzyme changes conformation by extending the PTS2 dimer towards LmPEX7 or by binding to any of the predicted β-propeller blades needs to be determined. It is also likely that the *Leishmania* PEX7 may bind a monomeric aldolase with an exposed PTS2 signal and that subsequent oligomerization of this enzyme occurs inside the glycosome. Although there is currently no crystal structure of the trypanosomatid PEX7, the predicted model of LmPEX7 gives insight into the potential binding sites and function of this protein. This structure will also serve as a template that can be tested biochemically.

The exposed hydrophobic residues on the surface of LmPEX7 could explain why the refolding of LmPEX7 required reducing conditions and the chemical chaperone glycerol to obtain a functional protein (Pilar, et al. in prep). Refolding of LmPEX7 generated a soluble protein but whether a chaperone is required for glycosomal protein import is unclear although the chaperones Hsp70 and DNAJ have been suggested to be important for peroxisome biogenesis [53-56].

Sequence analysis of LmPEX7 showed that the N-terminal domain contains the consensus motif PGX₂GX₆PX₁₀F/YGX₂GXGX₉G that is conserved among PEX7 proteins from trypanosomatids, yeast, and mammals while a poly-proline repeat found at the C-terminus is only present in trypanosomatid PEX7. Functional analysis of LmPEX7 fragments encoding the N- and C-terminal regions overexpressed in *E. coli* showed that only the N-terminal portion spanning amino acids 1-223 was important for binding.
LdPEX5 and His6/LdPEX14. The full-length protein, however, is needed for ldald 1-77-His6 binding activity. Similarly, the full-length PEX7 is required for binding PTS2 and PEX5 in yeast and mammals and 11-14 amino acid-truncations either at the N or C-terminus abrogated these protein-protein interactions [57, 58].

The ability of *Leishmania* PEX7 to target to the glycosomes was investigated by overexpressing LmPEX7-GFP fusion proteins in *L. donovani* promastigotes. Subcellular localization studies showed that the full-length LmPEX7-GFP protein had a predominantly glycosomal localization with a small proportion found in the cytosol. This protein was also capable of binding both PTS2 and LdPEX5 similar to the native *Leishmania* PEX7. In peroxisomes, the subcellular localization of PEX7 had been a matter of debate as the localization of this protein was affected by the type and position of the tag being used [59, 60]. In yeast, placing a tag at the C-terminus seemed to impair PEX7 recycling due to destabilization of this protein leading to increased protein degradation [58]. In our study, LmPEX7-GFP was stably expressed as shown in Western blot analysis of cell extracts from transfected parasites. These results showed that the GFP tag did not affect the localization of LmPEX7.

To determine the functional domains of LmPEX7 responsible for targeting to the glycosomes, the first 71 amino acids of LmPEX7 and the C-terminal domain (aa 223-373) were expressed as GFP fusion proteins in promastigotes. Immunofluorescence analysis of the transgenic promastigotes expressing lmpex7 1-71-GFP and lmpex7 CT-GFP showed a predominantly cytosolic localization. However, definitive subcellular localization of these LmPEX7 fragments by immunofluorescence could not be determined since the transgenic parasites expressed very low levels of GFP. It is possible that these LmPEX7 fragments are not stable, easily get degraded, and also promote the degradation of GFP. Subcellular fractionation of lysates from these transgenic promastigotes revealed that lmpex7 1-71-GFP and lmpex7 CT-GFP are found in the cytosolic fractions. This variable distribution could either be due to inefficient targeting to the glycosomes leading to a population of lmpex7 1-71-GFP and lmpex7 CT-GFP
proteins accumulating in the cytosol. The exposed hydrophobic residues on the mutant fragments could have also caused protein aggregation.

Protease protection assays of transgenic promastigotes showed that both Lmpex7 1-71-GFP and Lmpex7 CT-GFP were resistant to proteolysis. The light mitochondrial pellet obtained after spinning the postnuclear lysates at 40,000 x g and contained mostly mitochondria, lysosomes, and glycosomes, was used to specifically probe for glycosomal matrix proteins which are protected from proteolytic digestion. The results of the protease protection assays lead to the notion that these mutant proteins associated with the glycosomal matrix. However, it is also possible that the LmPEX7 mutant proteins could be inherently resistant to proteolysis. In mammals, a full-length PEX7 was required for targeting into peroxisomes while the first 56 amino acids at the N-terminal portion of the yeast PEX7 might contain the translocation signal [57, 58, 61].

Pull-down assays using LdpeX5NT and the PTS2 ligand Ldald 1-77 coupled to affinity beads did not precipitate Lmpex7 1-71-GFP or Lmpex7 CT-GFP similar to binding assays with the recombinant mutant proteins. These results further show that the mutant proteins were probably non-functional and unstable when expressed in parasites. The function of the conserved proline-glycine motif found within amino acids 1-71 of LmPEX7 still has to be determined as the presence of this motif in Lmpex7 1-71-GFP was not enough to bind LdpeX5NT.

How the LmPEX7 mutant proteins might associate with the glycosomes despite losing the ability to bind a PTS2 ligand or LdPEX5 is unknown. It is possible that these mutant proteins are being trafficked to the organelle by a “piggy-back” mechanism, a pathway observed in peroxisomes where proteins lacking a peroxisomal targeting signal sequence are imported into the organelle by associating with other peroxisomal proteins [62]. Generating LmPEX7 fragments with a different tag or applying alternative live fluorescence imaging techniques could be used to further examine the mechanism of Leishmania PEX7 targeting to the glycosome. Our lab is currently developing an inducible protein expression system to control the levels of LdPEX7, LdPEX5 or
LdPEX14 in *L. donovani* promastigotes in order to determine the effect on the targeting of *Leishmania* PEX7 and LdPEX14 to the glycosome and on the mechanism of protein translocation. This would also provide an alternative to RNAi and circumvent the problem of generating Δldpex5 or Δldpex14 null mutants, experiments which are not possible in *L. donovani* [30, 36]. The identification and characterization of novel glycosomal components involved in biogenesis will also have to be accomplished to further elucidate the steps in glycosome biogenesis.
Chapter 5 References


Chapter 6. Summary and conclusions

Knowledge of the function of the glycosome is crucial to the development of chemotherapeutic agents against this potential drug target. The field of glycosome biology is also important in studying highly divergent and medically important organisms like the trypanosomatids. Glycosomes are essential for the growth and survival of the parasites *Leishmania* and *Trypanosoma* and require a system of importing proteins involved in glycosome assembly and in the various metabolic processes housed in these organelles [1, 2]. Because glycosomes are specialized for glycolysis, proper sorting of PTS2 proteins such as the glycolytic enzymes hexokinase and aldolase is required. However, not much is known about glycosomal PTS2 trafficking in which the receptor PEX7 plays a pivotal role. To dissect the PTS2 glycosomal import pathway, the role of the PTS2 receptor LmPEX7 in *L. donovani* was investigated.

*Leishmania* PEX7 was characterized based on bioinformatic, biochemical, biophysical, and molecular data. It has to be noted that PEX7 in *L. major* and *L. donovani* share 97% sequence identity and that the antibodies raised against LmPEX7 also cross-reacted with the native form of PEX7 in *L. donovani*. The use of PEX7 in both species is therefore, interchangeable. LmPEX7 displays low sequence similarity to yeast and mammalian PEX7 homologs and contains unique N- and C-terminal extensions. The trypanosomatid PEX7 has polyproline repeats at the C-terminus which are absent in yeast and mammalian PEX7. The predicted structure of LmPEX7 shows a β-propeller fold consistent with that of other members of the WD40 family. However, due to the diversity of functions of the different WD40 members [3], the function of LmPEX7 cannot be inferred by structural similarity alone. The predicted structural model of LmPEX7 will ultimately have to be proven by crystallography and other biochemical techniques.

Initial investigation of the role of PEX7 in glycosomes and peroxisomes was previously hampered by the difficulty in expressing the protein in soluble form [4]. LmPEX7 expressed in *E. coli* was found in both inclusion bodies and in soluble form. However, the
soluble form of LmPEX7 co-purified with bacterial GroEL and various attempts were
done to dissociate LmPEX7 from this chaperonin. Despite the presence of GroEL, it did
not affect the activity of LmPEX7 which functioned similarly to that of the native
*Leishmania* PEX7, binding to PTS2 ligands and to LdPEX5 or LdPEX14 in protein
interaction assays. A homogeneous and soluble form of LmPEX7 was obtained by
purifying LmPEX7 from inclusion bodies and refolding the protein on a Ni$^{2+}$-NTA
column using a step down gradient of urea and glycerol. This protein remained soluble in
5% glycerol and retained its functional activity. The success in purifying a soluble form
of LmPEX7 provided vast opportunities to properly dissect the mechanism of PTS2
protein import in glycosomes.

Structural analysis of LmPEX7 showed that it forms predominantly tetrameric structures
in the presence of a PTS2 ligand, LdPEX5, and LdPEX14. The oligomeric structure of
this protein is not surprising as both LdPEX5 and LdPEX14 have also been shown to
form oligomeric structures [5]. Whether the oligomeric nature of LmPEX7 is inherent in
the proper functioning of this receptor *in vivo* needs further investigation. The structure of
the native *Leishmania* PEX7 can be further examined by performing glycerol density
gradient centrifugation of cell extracts from promastigotes under mild lysis conditions or
with the use of cross-linking agents. Moreover, changes in the secondary structure of
LmPEX7 upon ligand binding can be examined by circular dichroism.

Using *in vitro* and *in vivo* functional assays, the *Leishmania* PEX7 specifically bound
PTS2-containing proteins, aldolase and hexokinase, proving that it is a PTS2 receptor.
Because both aldolase and LmPEX7 have similar molecular masses, e.g. 40 kDa, a
fragment of aldolase, ldald 1-77, was used in most of the assays to differentiate the two
proteins. Nevertheless, both the full-length aldolase and ldald 1-77 showed similar
interaction with LmPEX7.

The PTS1 receptor LdPEX5 and the docking protein LdPEX14 are essential for the
sorting of proteins to the glycosome [6, 7]. Proper functioning of this organelle also
requires coordination between PEX5 and PEX7 [2]. How *Leishmania* PEX7 plays a role
in glycosome biogenesis and how it interacts with the components of the PTS1 import pathway were investigated using different molecular approaches. Using mutagenesis experiments, the LmPEX7 binding domains on LdPEX5 and LdPEX14 were identified which lead to the conjecture that both LdPEX5 and LdPEX14 are involved in the PTS2 import pathway. The nature of the interactions between LmPEX7 and the various components of the glycosomal import machinery was examined by molecular and biophysical techniques. An ELISA-based interaction assay was employed to quantitatively assess the changes in the binding affinities upon formation of glycosomal protein complexes. This gave insight into the possible mechanism of protein import into the organelle. Interestingly, both the PTS1 and PTS2 pathways have similar molecular dynamics. The LmPEX7-LdPEX5, LmPEX7-LdPEX14, and LdPEX5-LdPEX14 associations were modulated by the binding of the PTS cargo proteins to their receptors [5]. The LmPEX7-PTS2 binding affinity is increased upon binding of LdPEX5 to LmPEX7. Conversely, LmPEX7 does not readily bind a cargo-loaded LdPEX5 which suggests that the empty receptors probably form a complex in the cytosol prior to binding their respective cargo proteins. The formation of glycosomal protein complexes corresponded with structural changes in LmPEX7, LdPEX5, and LdPEX14 as demonstrated by changes in tryptic digestion profiles. Limited trypsin proteolysis will have to be performed to determine whether native protein complexes from *Leishmania* also display similar digestion patterns. To confirm the binding affinities obtained from ELISA-based interaction assays, other quantitative techniques like surface plasmon resonance, which determines binding affinities between proteins in real time, can be employed.

LmPEX7 contains ten tryptophan residues, seven of which are part of the WD40 motifs. Since tryptophan is a sensitive indicator of local and global changes in protein structure upon ligand binding [8], the structural changes in LmPEX7 induced by binding a PTS2 ligand or LdPEX5 were examined by fluorescence spectroscopy. These protein associations increased the intrinsic fluorescence of LmPEX7 which further corroborated results from the ELISA-based interaction and tryptic digestion experiments. Surprisingly, LmPEX7 displayed complex emission wavelength probably due to the different local
environments of the tryptophan residues which influence tryptophan fluorescence [9]. This was further confirmed by quenching experiments using acrylamide and potassium iodide. The interaction of LmPEX7 with a PTS2 ligand and LdPEX5 also corresponded with changes in the surface hydrophobicity of LmPEX7 as demonstrated in fluorescence studies using ANS. These studies provided an insight into the upstream events of protein import into the glycosomes and illustrated the dynamics of interactions between LmPEX7 and the other components of the glycosomal machinery.

Various models have been proposed to illustrate the mechanism of protein import into peroxisomes [10, 11]. Whether the PTS receptors function as shuttling receptors or whether PEX5 forms a transient pore on the membrane, has yet to be proven in trypanosomatid glycosomes. Based on subcellular localization studies, LdPEX5 does not associate with the glycosome and shows a predominantly cytosolic distribution. On the other hand, LmPEX7 may function as a shuttling receptor as it displays a bimodal distribution, localizing in the cytosol and in the glycosomes. However, the mechanism of LmPEX7 targeting to the glycosome needs to be further investigated as studies to determine the domains required for targeting of LmPEX7 were generally inconclusive. The truncated LmPEX7 mutants expressed in *L. donovani* were unstable and had anomalous subcellular localization. N- and C-terminal truncations in LmPEX7 abrogated the ability to bind PTS2 and LdPEX5, suggesting that a full-length LmPEX7 is needed for proper targeting and protein binding. The development of alternative fluorescent imaging techniques (FRET, use of other fluorescent tags, etc.) or an inducible protein expression system in *Leishmania* would further elucidate the binding domains on LmPEX7 and its targeting to the glycosome.

All the studies presented here contributed to the elucidation of the glycosomal PTS2 pathway in which the *Leishmania* PEX7 plays a key role in protein translocation. These studies also highlighted the interface between the PTS1 and PTS2 import pathways through the interaction of the receptors LmPEX7 and LdPEX5. In an effort to integrate the results obtained from these studies, a model illustrating the mechanism of glycosomal protein import is presented in Fig. 28. The upstream event in glycosomal protein
translocation involves the formation of the LdPEX5-LdPEX7 complex in the cytosol, mediated by a distinct LdPEX7 interaction domain on LdPEX5, prior to binding their respective PTS ligands. This association induces a structural change in LmPEX7 to an import-competent state with increased PTS2 binding affinity. The structural changes in both PTS receptors could also promote docking on the membrane-associated LdPEX14 through distinct receptor binding domains on LdPEX14. That LdPEX14 could be a major part of a glycosomal translocon is suggested by studies which show that this protein forms a multimeric complex on the glycosomal membrane and undergoes extensive structural changes upon binding LdPEX5 [5, 7, 12]. The structural changes induced in LdPEX14 upon complex formation and the decreased receptor-cargo binding affinity in the presence of LdPEX14 could be the triggers for the translocation of the LmPEX7-PTS2 complex into the glycosome and subsequent release of the PTS cargo protein into the matrix. In the case of the PTS1 import pathway, LdPEX5 binds a PTS1 ligand independent of that of LmPEX7 so that the LdPEX5-PTS1 complex formation takes place either before or after the formation of a docking complex. The mechanism of how the PTS1 protein gets imported into the glycosome without LdPEX5 entering the glycosomal matrix needs further investigation. It is possible that PTS1 translocation is aided by the structural changes induced in LdPEX14 that eventually modify the glycosomal membrane topology and promote PTS1 import. Alternatively, other peroxins involved in protein import which still need to be identified and characterized could play a role in protein translocation across the glycosomal membrane. Identification of these novel glycosomal peroxins by tandem affinity purification, proteomic analysis, and determination of physiologic factors (ATP, pH, temperature, etc.) that influence protein import will further enhance studies on the mechanism of glycosomal protein trafficking.

In conclusion, the mechanism of glycosome biogenesis in trypanosomatids is a dynamic process which involves formation of stable protein complexes and coordination of the PTS import pathways. The cross-talk between the two import pathways is exemplified by the crucial interaction of the two import receptor proteins and docking on LdPEX14 which represents the converging point of these two pathways. The salient steps in glycosomal protein import need to be studied not only to understand glycosome function
but also to identify possible drug targets in *Leishmania*. The development of inhibitors specific to the glycosome is possible due to sufficient differences between glycosomes and peroxisomes as shown by the low sequence similarity and distinct binding domains of glycosomal proteins and the differences in the mechanism of glycosomal biogenesis. Therefore, studies that further enhance our knowledge on glycosomes will prove useful in combating leishmaniasis and in understanding trypanosomatid biology.
Figure 28. Model of glycosomal protein import in *Leishmania*. (1) Empty LdPEX5 and LdPEX7 form a complex in the cytosol which is mediated by a distinct LdPEX7 binding domain on LdPEX5. (2) This association increases the binding affinity of LdPEX7 for its PTS2 ligand. (3) Corresponding structural changes in both receptors promote docking on the glycosomal membrane through LdPEX14. LdPEX14 undergoes structural changes which modulate the receptor-cargo protein binding affinity to trigger the translocation of LdPEX7-PTS2 into the glycosomal matrix. (4) LdPEX7 releases the PTS2 cargo into the matrix and recycles back to the cytosol (5). LdPEX5 binds a PTS1 ligand independent of LdPEX7 and targets the PTS1 cargo to the glycosomal membrane (*a, dotted arrow*). Alternatively, PTS1 interacts with LdPEX5 after formation of the docking complex (*b, dotted arrow*). The release of PTS1 into the glycosomal matrix could be mediated by structural changes in LdPEX14 which promote PTS translocation or through other glycosomal proteins. The lower part of the figure shows the binding sites on LdPEX5 and LdPEX14 for protein complex formation.
Chapter 6 References


Appendix 1. Permit from publishers

Copy of the letter of permission from publishers regarding manuscript reproduced in Chapter 3 Interaction of the *Leishmania* receptor peroxin 7 with the glycosomal import machinery.

Re: Request for permission
From: Jones, Jennifer (ELS-OXF) [J.Jones@elsevier.com]  
Sent: Monday, August 11, 2008 5:42 AM  
To: Ana Victoria Pilar, Ms  
Cc: Armando Jardim, Prof.  
Subject: RE: Request for permission

Dear Ana Victoria C. Pilar

As author of the requested article, you do not need Elsevier's permission to include it in your thesis as it is part of the author's rights you retain as an Elsevier journal author.

For further information on the rights you retain as an Elsevier journal author, please visit our web page at http://www.elsevier.com/wps/find/authorsview.authors/copyright.

Yours sincerely
Jennifer Jones  
Rights Assistant  
Global Rights Department

Elsevier Ltd  
PO Box 800  
Oxford OX5 1GB  
UK

Elsevier Limited, a company registered in England and Wales with company number 1982084, whose registered office is The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom.

Elsevier is pleased to announce our partnership with Copyright Clearance Center's Rightslink service. Rightslink will handle Elsevier's journal permission requests. For your future requests, you have to go through Rightslink to obtain permissions. With Rightslink (r) it's faster and easier than ever before to obtain permission to use and republish material from Elsevier. Using Rightslink is as simple as:

Simply visit: http://www.sciencedirect.com/ and locate your desired content.  
Click on the article.

Open the abstract or summary and then click on the 'Request Permission' button to open the Rightslink web page which will launch you into the Rightslink application and then follow the steps below.
To Whom It May Concern,

I would like to ask for permission to reproduce my paper published in Molecular and Biochemical Parasitology for my PhD thesis. It is a requirement by McGill University to attach permissions/waivers for published material included in the dissertation.

I tried to use RightsLink at Science Direct to obtain permission for the paper but I got a message saying that the content/journal cannot be loaded.

The article is:

Interaction of Leishmania PTS2 receptor peroxin 7 with the glycosomal protein import machinery
Ana Victoria C. Pilar, Kleber P. Madrid and Armando Jardim

Molecular and Biochemical Parasitology
Volume 158, Issue 1
March 2008, Pages 72-81

Sincerely,

Ana Victoria C. Pilar