Analysis and Expression of *Leishmania* Surface Proteinase and Glycoproteins

by

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Abstract

*Leishmania* is a protozoan parasite transmitted by a sandfly vector to humans and other mammalian hosts causing chronic infection. *Leishmania* exists in two distinct life stages: the promastigote which replicates in the insect gut, and the amastigote which replicates in macrophage cells of the mammalian host. There is considerable evidence that *Leishmania* cell surface molecules play critical roles in the initiation and establishment of macrophage infection, evasion of non-specific immune defenses, and in stimulation of a cellular and humoral immune response. Relative to promastigotes, the amastigote cell surface is largely uncharacterized. The major surface glycoprotein of *Leishmania* promastigotes, referred to as GP63, is a zinc metalloproteinase of 63,000 Mr containing a glycosylphosphatidylinositol (GPI) membrane anchor. GP63 has been predicted to be synthesized as a 602 amino acid precursor protein containing an NH2-terminal leader sequence of 39 amino acids, a putative regulatory pro region of 61 amino acids, the mature protein of 477 amino acids, and a COOH-terminal GPI attachment signal sequence of 25 amino acids. Recent studies demonstrated that recombinant GP63 (rGP63) expressed by the baculovirus insect cell system was secreted as a glycosylated latent proteinase that required activation for full proteinase activity (Button, L.L. *et al. Gene* 134:75-81, 1993). The overall objectives of this study were to determine the activation mechanism of recombinant GP63 (rGP63), using both secreted and cell surface expression systems, and to identify novel glycoproteins expressed on the surface of *L. mexicana* amastigotes.

The activation of secreted latent rGP63 (rGP63s) was found to be consistent with the cysteine switch mechanism described for mammalian matrix metalloproteinases. NH2-terminal sequence analysis revealed that activation of rGP63s with HgCl2 resulted in progressive autolytic removal of the predicted pro region, ultimately generating the mature NH2-terminus. This processing included the removal of a conserved Cys residue (Cys-48) and occurred by a cis mechanism since the addition of previously activated rGP63s did not
lead to an enhancement of latent rGP63s proteinase activation. rGP63s expressed in COS-7 cells was also secreted as a latent proteinase and appeared to be activated by the same mechanism. rGP63 expressed on the surface of COS-7 cells as either a GPI-linked protein (rGP63gpi) or as a transmembrane fusion protein (rGP63tm) was produced as an active proteinase without the requirement for activation with HgCl$_2$. Expression of GP63 as an active proteinase, therefore, is not linked specifically to the GPI pathway and does not require a factor or microenvironment unique to *Leishmania*.

The potential of glycoprotein purification to enrich for cell surface proteins was exploited in a search to identify novel amastigote surface molecules. The strategy was to isolate candidate glycoproteins, by lectin affinity chromatography and SDS-PAGE, for NH$_2$-terminal amino acid sequence analysis in order to design oligonucleotide probes to isolate a corresponding cDNA clone. The most abundant glycoproteins were identified by NH$_2$-terminal sequence analysis as Cys proteinases, GP63, and fragments thereof. Of 12 sequences obtained, 5 appeared to be unique and provide a basis for further studies to isolate the corresponding genes.
Table of Contents

Abstract ................................................................. ii
Table of Contents ........................................................... iv
List of Tables .............................................................. vii
List of Figures .............................................................. viii
Abbreviations ............................................................. x
  A. general ............................................................... x
  B. GP63 gene constructs and recombinant protein ..................... xii

Chapter 1. Introduction .................................................. 1
  A. An Overview of Leishmaniasis .................................. 1
  B. The Leishmania Life Cycle .................................. 2
  C. Leishmania Cell Surface Proteins and Lipophosphoglycan .... 4
     (1) the promastigote cell surface .......................... 4
     (2) the amastigote cell surface ............................ 7
  D. The Host Immune Response to Leishmania ....................... 8
     (1) cellular immunity mediated by a Th1/Th2 dichotomy .... 8
     (2) immune effector mechanisms ........................... 14
     (3) innate resistance in murine leishmaniasis ............ 16
  E. Evasion of Non-specific Host Defenses ......................... 17
     (1) complement fixation and serum resistance .......... 18
     (2) promastigote - macrophage interactions: attachment and phagocytosis .... 19
     (3) survival within the macrophage phagolysosomal compartment .... 23
  F. The Leishmania Surface Proteinase GP63 and its Similarity to Mammalian .... 25
     Matrix Metalloproteinases
     (1) GP63 ....................................................... 25
     (2) matrix metalloproteinases and the extracellular matrix ..... 28
  G. Objectives of this Study ......................................... 34
     (1) analysis of the activation mechanism and processing of GP63 .......... 34
     (2) identification of novel amastigote glycoproteins ................ 35
Chapter 2. Materials and Methods ......................................................... 36

A. Leishmania ......................................................................................... 36
   (1) maintenance of Leishmania promastigotes in vitro ....................... 36
   (2) maintenance of L. mexicana amastigotes in vitro ......................... 36
   (3) isolation of L. mexicana lesion amastigotes .................................. 36

B. Preparation of Rabbit Antisera ......................................................... 37
   (1) production and affinity purification of a rabbit antiserum (anti-prepro) .... 37
      raised against the GP63 prepro peptide
   (2) production of an anti-amastigote rabbit serum and absorption with ...... 3
      promastigotes

C. Biotinylation of Amastigotes and Promastigotes ............................... 38

D. Protein Purification ........................................................................... 39
   (1) affinity purification of rGP63s ..................................................... 39
   (2) glycoprotein purification using concanavalin A and lentil lectin ......... 40
      affinity chromatography
   (3) fractionation of purified glycoproteins by differential EtOH precipitation .. 41
   (4) preparation of proteins for NH2-terminal amino acid sequence analysis ... 41
      by SDS-PAGE and electroblot transfer to Immobilon membrane

E. Activation of rGP63s and Determination of Proteinase Activity .......... 42

F. Expression of rGP63 in COS-7 Cells .................................................. 44
   (1) PCR-modification of GP63 for expression in COS-7 cells ............... 44
   (2) transfection of COS-7 cells and preparation of lysates ................. 46
   (3) fluorescence flow cytometry analysis .......................................... 47

G. Analytical SDS-PAGE and Western Blot Analysis ............................ 47

Chapter 3. Analysis of the Activation Mechanism and Processing .......... 49
            of rGP63

A. Results .............................................................................................. 49
   (1) activation and autocatalytic processing of rGP63s ...................... 49
      (a) affinity purification of rGP63s ................................................. 49
      (b) autolytic activation of rGP63s proteinase activity ...................... 51
      (c) progressive removal of the rGP63s pro region upon activation .... 58
         with HgCl2
(2) processing of secreted and membrane rGP63 expressed in COS-7 cells .... 61
   (a) rGP63s is secreted from COS-7 cells as a latent proteinase ........... 61
   (b) wildtype GP63 (rGP63gpi) is expressed as an active proteinase ...... 68
       on the surface of COS-7 cells
   (c) a transmembrane fusion protein of GP63 (rGP63tm) is expressed ..... 71
       as an active proteinase on the surface of COS-7 cells

B. Discussion .............................................................................................................. 79

Chapter 4. Identification of Novel Amastigote Glycoproteins .......................... 90

A. Results ...................................................................................................................... 91
   (1) suitability of an L. mexicana in vitro amastigote model for .......... 91
       characterization of amastigote proteins
   (2) affinity purification of in vitro amastigote cell surface glycoproteins ...... 94
       by concanavalin A-sepharose affinity chromatography
   (3) NH2-terminal amino acid sequence analysis of in vitro amastigote ...... 97
       glycoproteins

B. Discussion .............................................................................................................. 104

Chapter 5. General Discussion ............................................................................... 109

Appendix. RT-PCR Generation of Amastigote cDNA and PCR .................. 112
   Amplification Using a Primer Specific for the 43 kDa Glycoprotein

A. Experimental Procedures ................................................................................. 112
   (1) purification of total RNA from lesion amastigotes and DNaseI treatment .. 112
   (2) cDNA synthesis: reverse transcription and PCR .................................. 112

B. Results .................................................................................................................... 115

References .................................................................................................................. 120
List of Tables

Table 1  The matrix metalloproteinase family .................................................. 29
Table 2  Alignment of GP63 sequences surrounding the proposed regulatory ...... 83
cysteine
Table 3  Similarity of GP63 to the matrix metalloproteinase family .................. 85
Table 4  Alignment of the GP63 active site with the MMP and astacin family ...... 87
consensus sequences
Table 5  Summary of NH2-terminal amino acid sequence analysis of ............... 101
glycoproteins from *L. mexicana in vitro* amastigotes purified by conA
lectin affinity chromatography and SDS-PAGE
Table 6  Summary of NH2-terminal amino acid sequence analysis of ............... 102
glycoproteins from *L. mexicana in vitro* amastigotes purified by lentil
lectin affinity chromatography and SDS-PAGE
List of Figures

Figure 1  The *Leishmania* life cycle .................................................. 3
Figure 2  The structure of *Leishmania* lipophosphoglycan (LPG) ................. 5
Figure 3  The cytokine network in *L. major* infections .............................. 10
Figure 4  Schematic diagram of predicted post-translational processing of *Leishmania* GP63 26
Figure 5  The domain structure of MMPs .................................................. 30
Figure 6  The cysteine switch mechanism ................................................ 33
Figure 7  Affinity purification of rGP63s from baculovirus supernatant ........... 50
Figure 8  Activation of purified latent rGP63s by HgCl2 and inhibition by ....... 52
1,10-phenanthroline
Figure 9  Proteinase activity of HgCl2-activated rGP63s is comparable to ....... 54
native promastigote GP63
Figure 10 Time course of rGP63s proteinase activity and autolytic activation ...... 55
of rGP63s
Figure 11 NH2-terminal amino acid sequence of latent and HgCl2-activated ...... 59
rGP63s
Figure 12 Schematic diagram of GP63 constructs modified for surface .......... 62
expression or secretion in COS-7 cells
Figure 13 COS-7 cell expression of either the wtGP63-S gene or the GP63-S ...... 63
gene results in secretion of glycosylated rGP63s
Figure 14 HgCl2 activation of rGP63s produced in COS-7 cells .................... 65
Figure 15 Western blot analysis using an anti-prepro rabbit serum and a mAb ...... 67
specific for promastigote GP63
Figure 16 Cell surface expression of rGP63gpi on transfected COS-7 cells ........ 69
Figure 17 rGP63gpi produced in COS-7 cells is glycosylated .......................... 70
Figure 18 rGP63gpi is produced as an active proteinase in COS-7 cells .......... 72
Figure 19 Western blot analysis using a mAb specific for the VSV-G ............ 74
transmembrane region
Figure 20  rGP63tm and rGP63gpi are expressed at similar levels on transfected COS-7 cells

Figure 21  EndoF analysis of rGP63tm produced in COS-7 cells

Figure 22  Effect of HgCl2 treatment on membrane versus secreted rGP63 produced in COS-7 cells

Figure 23  Differential cell surface labeling of promastigotes and in vitro amastigotes with an anti-amastigote rabbit serum

Figure 24  Antigenic differences between GP63 expressed in L. mexicana promastigotes and in vitro amastigotes

Figure 25  Cell surface expression of GP63 on in vitro amastigotes and enrichment with conA

Figure 26  ConA affinity purification of biotin-labeled glycoproteins isolated from surface labeled in vitro amastigotes

Figure 27  ConA and lentil lectin affinity purification of in vitro amastigote glycoproteins and further fractionation by differential EtOH precipitation

Figure 28  Protein sequence alignments of amastigote glycoprotein NH2-terminal sequences with published sequences

Figure 29  Schematic diagram of L. mexicana Cys proteinase and the 43 kDa, 16 kDa and 13 kDa glycoproteins purified from L. mexicana in vitro amastigotes

Figure 30  Primer strategy for RT-PCR generation of an amastigote cDNA library

Figure 31  First and second strand cDNA synthesis using RT-PCR

Figure 32  Sequence and design of MH43K 3' primer for PCR amplification of a cDNA fragment encoding a 43 kDa conA+ amastigote glycoprotein

Figure 33  DNA fragments obtained by PCR amplification of amastigote and promastigote cDNA using the 5'SL primer and the 3'MH43K primer
Abbreviations

A. general

aa  amino acid
BCIP  5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt
BSA  bovine serum albumin
cDNA  complementary deoxyribonucleic acid
CL  cutaneous leishmaniasis
conA  concanavalin A
CPM  counts per minute
dATP  deoxyadenosinetriphosphate
DCL  diffuse cutaneous leishmaniasis
dCTP  deoxycytodinetriphosphate
DEPC  diethyl pyrocarbonate
dGTP  deoxyguanosinetriphosphate
DMSO  dimethylsulfoxide
DNA  deoxyribonucleic acid
DNAse I  deoxyribonuclease I
DTT  dithiothreitol
dTTP  deoxythymidinetriphosphate
E64  trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane
ECM  extracellular matrix
EndoF  endoglycosidase F
EtOH  ethanol
FCS  fetal calf serum
FITC  fluorescein isothiocyanate
GM-CSF  granulocyte macrophage colony stimulating factor
GPI  glycosylphosphatidlyinositol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthetase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LPG</td>
<td>lipophosphoglycan</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCL</td>
<td>mucocutaneous leishmaniasis</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type - matrix metalloproteinase</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NMMA</td>
<td>N-monomethylarginine</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyisulfonyl fluoride</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription - polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
</tbody>
</table>
TNF  
tumour necrosis factor

Tris  
tris(hydroxymethyl)aminomethane

uPA  
urokinase type plasminogen activator

VL  
visceral leishmaniasis

VSV-G  
vesicular stomatitis virus glycoprotein G

B. GP63 gene constructs and recombinant protein
(see Materials and Methods for detailed descriptions)

rGP63  
recombinant GP63 protein

rGP63s  
recombinant secreted GP63 protein

rGP63gpi  
recombinant GPI-linked GP63 protein

rGP63-tm  
recombinant GP63 transmembrane fusion protein

wtGP63-GPI  
wildtype GP63 gene construct encoding GPI-linked GP63 protein (rGP63gpi)

GP63-GPI  
GP63 gene construct encoding GPI-linked GP63 protein (rGP63gpi) containing a modified NH2-terminal leader sequence

wt GP63-S  
GP63 gene deletion construct encoding a secreted form of GP63 protein (rGP63s) containing the wildtype NH2-terminal leader sequence

GP63-S  
a GP63 gene deletion construct encoding a secreted form of GP63 protein (rGP63s) containing a modified NH2-terminal leader sequence

GP63-TM  
a GP63 gene construct encoding a GP63 transmembrane fusion protein (rGP63tm) containing a modified NH2-terminal leader sequence
Chapter 1. Introduction

A. An Overview of Leishmaniasis

*Leishmania* is a protozoan parasite that is the causative agent of a complex group of human diseases termed leishmaniasis. The parasite is transmitted by phlebotomine sandflies to humans and animal reservoirs and replicates as obligate intracellular amastigotes within host macrophage cells. The occurrence of leishmaniasis is limited to tropical and sub-tropical climates by the range of the sandfly vector. In 1990, the World Health Organization estimated that there were 12 million cases of leishmaniasis occurring in 80 countries in Central and South America, Africa, Southern Europe and Asia (Modabber, 1990).

Leishmaniasis can be divided into four main disease categories caused by different *Leishmania* species: cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Pearson, 1984). CL, the most common disease form, is characterized by self-limiting skin lesions which are localized to the initial site of infection and heal within a few months in the absence of treatment. CL is caused principally by *L. major*, *L. tropica* and *L. infantum* in the Old World and by *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. pifanoi*, and *L. braziliensis* in South America. DCL, which is caused by *L. mexicana* and *L. amazonensis* in South America, is characterized by progressive spreading of cutaneous lesions extending from the initial site of infection. Once established, DCL does not regress without treatment. MCL, which is caused most frequently by *L. braziliensis*, begins as a simple cutaneous lesion which may heal within a few weeks or months. However, soon after the initial infection or even years later, metastases to the nasal mucosa occurs causing significant tissue destruction of the nose, mouth and pharynx. VL, also known as Kala-azar, is characterized by metastasis to macrophages and monocytes in the spleen, liver,
bone marrow, lymph nodes, and skin; unless treated, VL is usually fatal. VL is most prominent in India and Africa, where it is caused by *L. donovani* but VL also occurs in South America where it is caused by *L. chagasi*. The pathology of leishmaniasis is considered to be largely immune related.

B. The *Leishmania* Life Cycle

*Leishmania* exists in two distinct life stages: the promastigote which replicates in the insect gut, and the amastigote, which replicates in macrophages within the mammalian host (Figure 1). Amastigotes are transmitted to the female sandfly following a blood meal from an infected mammalian host. Within the insect gut, amastigotes differentiate into motile, uniflagellar promastigotes with various morphologies. Non-infective procyclic promastigotes (procyclics) appear within 24 hours and divide and differentiate over a 2-5 day period ultimately generating infective metacyclic promastigotes (metacyclics) which detach from the insect gut epithelium and move anterior to the cuticle-lined foregut (Sacks and Perkins, 1985; for review see Killick-Kendrick, 1990; and Bates, 1994a). Developmental modifications in terminally exposed sugar side chains of a promastigote surface molecule, lipophosphoglycan (LPG), are thought to mediate the lack of adherence exhibited by metacyclic promastigotes (Pimenta *et al.*, 1992; Sacks *et al.*, 1994). During a blood meal, infectious metacyclic promastigotes are deposited by the sandfly into the skin of the mammalian host and are phagocytosed by dermal macrophages (Locksely *et al.*, 1988). The phagosome of the macrophage fuses with lysosomes forming a phagolysosome compartment wherein the promastigotes differentiate to amastigotes within 24 hours post-infection, thereby completing the cycle (Lewis and Peters, 1977; Berman *et al.*, 1979).
Figure 1 The *Leishmania* life cycle

In the human host, infection begins when promastigotes are injected into the skin by the bite of an infected sandfly (1). Promastigotes are phagocytosed by macrophages (2) wherein they differentiate to amastigotes and multiply. Lysis of infected cells leads to release of further amastigotes which then infect new cells. While in passage in the blood, the infected cells may be picked up by the sandfly vector during feeding. After transformation to non-infective, procyclic promastigotes (5), reproduction and differentiation to infective metacyclic promastigotes occurs in the midgut of the insect (5) followed by migration to the proboscis (7). This figure was taken from Roitt et al, 1989.
In vitro models of both procyclic and metacyclic promastigotes can be obtained in culture. Differential binding to peanut agglutinin (PNA) has been exploited to obtain uniform populations of *L. major* and *L. donovani* procyclic promastigotes (PNA⁺) from logarithmic-phase cultures and metacyclic promastigotes (PNA⁻) from stationary phase cultures (Sacks *et al.*, 1985; Howard *et al.*, 1987). Homogeneous cultures of *L. mexicana* metacyclics are obtained by growth in acidic pH (Bates and Tetley, 1993).

**C. Leishmania** Cell Surface Proteins and Lipophosphoglycan

(1) the promastigote cell surface

The two best characterized promastigote surface molecules are LPG and GP63, the major surface glycoprotein (Bouvier *et al.*, 1987). LPG forms a densely packed glycocalyx on the promastigote surface and is present in over a million copies per cell (Pimenta *et al.*, 1991; Bahr *et al.*, 1993). The structure of LPG contains 4 domains: (1) a phophatidy/(myo)inositol membrane anchor, (2) a hexasaccharide glycan core, (3) a repeating polymer of phosphodiester linked disaccharide units, and (4) a small neutral oligosaccharide cap (Figure 2) (Orlandi, *et al.*, 1987; Turco *et al.*, 1987; McConville *et al.*, 1990; Ilg *et al.*, 1992 and Thomas *et al.*, 1992). Whereas the glycan core and membrane anchor are highly conserved, the disaccharide repeats exhibit extensive inter-species polymorphisms with respect to the number and content of saccharide side chains. The terminal cap is also polymorphic. GP63 is a 63,000 Mr glycoprotein also containing a glycosylphosphatidylinositol (GPI) membrane anchor and has been characterized as a zinc metalloproteinase (Etges *et al.*, 1986a; Bouvier *et al.*, 1989). GP63 is the major surface protein on promastigotes, present in approximately half a million copies per cell and constituting about 1% of the total cellular protein (Bouvier *et al.*, 1987; Bahr *et al.*, 1993).
Figure 2  The structure of *Leishmania* lipophosphoglycan (LPG)

* taken from Descoteaux and Turco, 1993
A variety of membrane associated enzyme activities and transporters have also been demonstrated functionally on the promastigote surface although most have not yet been characterized at the molecular level. Identified active transporters include the following: (1) a Ca\textsuperscript{2+}-dependent ATPase which may represent the surface H\textsuperscript{+}ATPase in \textit{L. donovani} that extrudes protons from the parasite (Meade \textit{et al.}, 1987, 1989 and 1991); (2) a D-glucose -H\textsuperscript{+} symport identified in \textit{L. donovani} and \textit{L. mexicana} (Zilverstein \textit{et al.}, 1986; Langford \textit{et al.}, 1994); (3) a H\textsuperscript{+}-L-proline cotransporter in \textit{L. donovani} (Zilverstein and Dwyer, 1985); and (4) a folate transporter identified in \textit{L. major} and \textit{L. donovani} (Ellenberger and Beverley, 1987). Passive transporters mediating facilitated diffusion of ribose and nucleosides have also been demonstrated (Pastakia and Dwyer, 1987; Aronow \textit{et al.}, 1987). Several enzyme activities have also been reported on the surface of promastigotes: (1) a 43 kDa 3'-nucleotidase and a 72 kDa 5'-nucleotidase identified on \textit{L. donovani} promastigotes (Dwyer and Gottlieb, 1984; Gottlieb and Dwyer, 1983); (2) cell surface acid phosphatases demonstrated on the surface of \textit{L. donovani} and \textit{L. mexicana} (Remaley \textit{et al.}, 1985; Menz \textit{et al.}, 1991); and (3) phosphatidylethanolamine-specific phospholipases on \textit{L. donovani} (Dwyer, 1987). A putative G protein has also been described in \textit{L. donovani} (Cassel \textit{et al.}, 1991).

A number of cell surface molecules have also been identified immunologically but most have not been well characterized. A 46 kDa membrane glycoprotein, GP46/M-2, was isolated and cloned from \textit{L. amazonensis} promastigotes and shown to elicit a protective immune response against infection in susceptible mice (Champs \textit{et al.}, 1988, Lohman \textit{et al.}, 1990). However, the function of this protein is unknown. Similarly, a family of \textit{L. major} proteins referred to as the promastigote surface antigen-2 complex was identified by antibodies raised against \textit{L. major} (Murray \textit{et al.}, 1989). LPG-associated proteins have also been identified and were shown to stimulate human T cells from patients with leishmaniasis (Russo \textit{et al.}, 1992; Kemp \textit{et al.}, 1993). Again, the function of these proteins is unknown.
Recently, a family of differentially expressed genes was cloned in *L. major*. One gene referred to as Gene B was shown to encode a hydrophilic protein expressed on the surface of infective metacyclic promastigotes and amastigotes but not on non-infective procyclic promastigotes (Flinn et al., 1994). It appears that the Gene B protein may be associated with LPG and glycosylphosphatidylinositol on the parasite cell surface. This is the first report of a surface protein marker for infective stages of *Leishmania* parasites.

(2) the amastigote cell surface

The amastigote cell surface remains relatively uncharacterized. Whereas promastigotes are readily obtained in culture, *in vitro* amastigote models have only recently been developed for a few South American species of *Leishmania*. Therefore, most of the identified cell surface proteins were first characterized in promastigotes.

Cell surface GP63 appears to be substantially down regulated on *L. mexicana* amastigotes, relative to promastigotes, and the majority of GP63 appears to be a soluble form localized to megasomes (Bahr et al., 1993). Furthermore, some strains of *Leishmania* may not express cell surface GP63 in the amastigote stage (Schneider et al., 1992). Similarly, the cell surface glycolipid LPG, which is expressed at 1-3 million copies per cell in promastigotes of *L. major, L. donovani* and *L. mexicana*, is substantially down-regulated at least 1000 fold in the amastigote stage (Bahr et al., 1993). However, there is evidence for an LPG-like molecule in the flagellar pocket of lesion *L. mexicana* amastigotes (Bahr et al., 1993) and an amastigote-specific LPG has been described for *L. major* (Turco and Sacks, 1991; Glaser et al., 1991; Moody et al., 1991).

Some of the transporters and enzymes described for promastigotes have also been reported in amastigotes. *L. donovani* amastigotes express 3' and 5' nucleotidases (Dwyer and Gottlieb, 1984; Dwyer, 1987; Gottlieb and Dwyer, 1983) and appear to have enhanced expression of an ATPase gene which may encode a surface H+-ATPase which extrudes protons from the parasite (Meade et al., 1989). *L. mexicana* axenic amastigotes express a
glucose transporter with an acidic pH optimum (Langford et al., 1994) and also express cell surface acid phosphatase (Menz et al., 1991). As described above, L. major amastigotes express the hydrophilic gene B protein recently identified on the surface of infective stages of L. major (Flinn et al., 1994). However, the function of this protein is not known. An amastigote stage-specific gene referred to as A2 has also been cloned recently from L. donovani and is predicted to encode a 22 kDa secreted protein although the cellular localization has yet to be determined (Charest and Matashewski, 1994).

The recent development of axenic amastigote culture conditions may facilitate further characterization of amastigote surface molecules. Amastigote-like organisms can be grown in axenic cultures for L. pifanoi, L. panamensis, L. braziliensis and L. mexicana and may provide a source of proteins expressed in vivo (Pan et al., 1984; Eperon and McMahon-Pratt, 1989; Bates et al., 1992 and 1994b) Analysis of the amastigote cell surface may lead to a better understanding of persistence of the amastigote within the host macrophage and may also lead to identification of antigens important to the host immune response to Leishmania. Amastigote surface molecules could be important targets for both chemotherapeutic and vaccine development.

D. The Host Immune Response to Leishmania

(1) cellular immunity mediated by a Th1/Th2 dichotomy

Leishmania parasites evade the host immune response by residing and multiplying within macrophages which normally kill phagocytosed micro-organisms. However, in self-limiting CL, a specific cell mediated immune response ultimately leads to activation of infected macrophages and killing of intracellular amastigotes. Although a humoral immune response is mounted, acquired immunity in resistant mouse strains is transferred by CD4+ T cells not by B cells (Liew et al. 1984, 1986). Anti-leishmanial antibodies are reported to
trigger complement mediated lysis of promastigotes *in vitro* and to enhance phagocytosis (Pearson and Steigbigel, 1980; Herman *et al.*, 1980) but there is little evidence for a significant corresponding role *in vivo*. Although serum from Kala-azar patients has been shown to contain high titres of antibodies which immunoprecipitate *L. donovani* promastigote surface proteins, LPG appears to block surface binding of immune sera to *L. donovani* promastigotes (Karp *et al.*, 1991). It is therefore unlikely that the humoral response in VL provides immunity to reinfection.

Sub-cutaneous injection of susceptible Balb/c mice with *L. major* promastigotes leads to a progressive disseminating infection which visceralizes and ultimately results in death whereas infection of "resistant" C57/BL, C3H/HeN or CBA mice with *L. major* leads to self-limiting cutaneous infection and resistance to re-infection. It has been demonstrated that activation of Th1 CD4+ T cells promotes cure of *L. major* infection in resistant mice whereas activation of Th2 CD4+ T cells leads to exacerbation of disease in susceptible strains. Protective immunity is adoptively transferred by Th1 cell lines isolated from resistant mice and exacerbation is transferred by Th2 lines isolated from susceptible mice (Scott *et al.*, 1988; Muller and Louis, 1989). Similarly, a resistant or susceptible phenotype can be reconstituted in SCID mice with *L. major* specific Th1 and Th2 cell lines isolated from resistant and susceptible strains, respectively (Holaday *et al.*, 1991).

Th1 and Th2 cells secrete mutually antagonistic cytokines which trigger different immune effector mechanisms (see Figure 3). Murine Th1 cells secrete IL-2, IFN–γ and TNF-β and promote a delayed type hypersensitivity response whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and promote a largely humoral immune response (Cher and Mosmann, 1987; Mosmann *et al.*, 1986; Scott and Kaufmann, 1991). Both Th subsets have been reported to secrete IL-3, GM-CSF, and TNF-α (Mosmann *et al.*, 1986, Cher and Mosmann, 1987; Cherwinski *et al.*, 1987). Human CD4+ T cell subsets corresponding to murine Th1 and Th2 cells have also been described and produce a similar pattern of cytokine secretion; in humans, however, Th1 and Th2 cells both secrete IL-10 which
Figure 3 The cytokine network in *L. major* infections

*L. major* promastigotes are phagocytosed by macrophages (MΦ) and Langerhans cells (LC), which present leishmanial antigens to T cells. The development of T helper cell precursors (Th0 cells) into IFN-γ and IL-2-producing Th1 cells is governed by (a) macrophage-derived IL-12; (b) the secretion of IFN-γ by natural killer (NK) cells early after infection, which is triggered by IL-2, IL-12, and IL-13; and (c) by the presence of soluble IL-4 receptor (sIL-4R), which inhibits the bioactivity of (Th0 cell-derived?) IL-4. In contrast, Th0 will differentiate into Th2 cells if there is a lack of sIL-4R, IL-12, and/or NK-cell-derived IFN-γ. Macrophage-derived IL-1 as well as B cells and/or B-cell-derived IL-2 are also implicated in this process. TNF-α, IFN-γ, IL-4, IL-7, and migration inhibitory factor (MIF) synergistically activate macrophages for killing of intracellular *Leishmania* via induction of NO, reactive oxygen intermediates (ROI) and TNF-α. TGF-β, IL-10 and, under certain conditions IL-4, are able to antagonize these effector pathways (IL-13 might have similar activity but it has not yet been shown in the murine system). The macrophage-deactivating effect of IL-10 can also result from suppression of both Th1 development and Th1 cytokine secretion. → denotes stimulation/induction, —|— denotes inhibition. This figure and legend was taken from Bogdan et al, 1993.
inhibits cytokine production of both human Th subsets (Del Prete et al., 1991 and 1993; Haanen et al., 1991; Parronchi et al., 1991; Wierenga et al., 1990).

Th1 and Th2 cells are thought to arise from a common precursor, Th0, with a mixed cytokine profile including IL-2, IFN-γ, IL-4 and IL-5 (Firestein et al., 1989; Street et al., 1990; Swain et al., 1990). IFN-γ and IL-12 are thought to be important in the induction of a Th1 type response whereas IL-4 and IL-2 invoke a Th2 type response. Furthermore, IL-4 and IL-10 appear to inhibit cytokine production of murine Th1 cells. A recent analysis of cytokine induction over the course of *L. major* infection in susceptible and resistant mice supports this hypothesis (Reiner et al., 1994). A population of CD4+ cells containing transcripts for IL-2, IL-4, IFN-γ plus or minus IL-10, appeared in the draining lymph nodes of all strains of mice on day 2 and the levels of IL-4 and IL-2 peaked on day 4 after infection. On day 7, the transcripts for IL-2, IL-4 and IL-10 were markedly reduced in resistant mice and returned to basal levels by day 14. In susceptible mice IL-4 continued to be expressed at high levels on day 7 and day 14 while IL-2 and IL-10 were slightly decreased. In resistant mice, a steady increase in IFN-γ levels from day 4 to day 14 was coincident with a sharp drop in IL-10 between day 2 and day 7. IL-12 appeared in both strains on day 7 consistent with observations that amastigotes stimulate macrophage production of IL-12 *in vitro* whereas promastigotes do not. IL-12 is secreted by monocytes and B cells and is reported to be a potent inducer of T cell and NK cell IFN-γ production (Manetti et al., 1994; Chan et al., 1992; Tripp et al., 1993) The apparent ability of promastigotes to avoid IL-12 induction of high levels of IFN-γ may therefore promote parasite survival during the initial stages of infection by avoiding macrophage activation.

NK cells have also been implicated in induction of a Th1 response to *Leishmania* by secretion of IFN-γ early in the course of infection (Scharton et al., 1993; Laskay et al., 1993; Young and Ortaldo, 1987) In C57BL/6 mice it was demonstrated that on day 4 after *L. major* infection NK cells were the primary source of IFN-γ production, thought to be induced by IL-2 and IL-13 prior to induction of IL-12 (Reiner et al., 1994). However,
by day 14 of infection CD4+ T cells were the main source of IFN-γ (Reiner et al., 1994). In mice with acquired resistance to *L. major* and *L. donovani*, IFN-γ production by *Leishmania*-specific CD8+ T cells is also thought to contribute to induction of a protective secondary response (Murray et al., 1992; Muller et al., 1994).

The importance of IFN-γ in induction of a protective Th1 response has also been demonstrated *in vivo*. Treatment of resistant C3H/HeN mice with anti-IFN-γ antibodies within two days following *L. major* infection abrogated resistance and resulted in a disseminating infection similar to that of Balb/c mice (Belosevic et al., 1989; Leiby et al., 1993). In contrast, treatment with anti-IFN-γ three or more days after infection had no effect. Furthermore, C57BL/6 derived-mice homozygous for a homologous disruption of the IFN-γ gene were rendered susceptible to *L. major* infection whereas wildtype and heterozygous littermates remained resistant (Wang et al., 1994). Treatment of resistant mice with anti-IL-12 antibody also exacerbated disease (Sypek et al., 1993). Conversely, injections of recombinant IL-12 initiated a protective Th1 response in Balb/c mice when administered daily during the first week of *L. major* infection (Sypek et al., 1993; Heinzel et al, 1993b). A single injection of anti-IFN-γ antibody abrogated the protective effect of recombinant IL-12 and restored the Th2 response. However, treatment with recombinant IFN-γ alone does not protect Balb/c mice. Thus, although the effect of IL-12 appears to be IFN-γ dependent, other IL-12 induced factors may also be involved.

Treatment of susceptible Balb/c mice with anti-IL-4 antibodies at the time of infection with *L. major* led to a protective T cell response which was adoptively transferred to syngeneic irradiated recipients (Sadick et al., 1990). Furthermore, whereas CD4+ T cells in draining lymph nodes of infected Balb/c mice were shown to express high levels of IL-4 and IL-10 mRNA, mice treated with anti-IL-4 antibody at the time of infection displayed CD4+ T cells with increased IFN-γ and reduced IL-4 and IL-10 expression (Heinzel et al, 1991). CD4+ T cells from resistant C57BL/6 mice infected with *L. major* expressed significant levels of IFN-γ and IL-2 mRNA and barely detectable levels of IL-4
and IL-10 mRNA (Heinzel et al., 1991; Morris et al., 1993). In both resistant and susceptible strains, B cells in the draining lymph nodes were found to express IL-2 mRNA (Heinzel et al., 1991). In vitro, IL-2 in combination with IL-4 was shown to be necessary for induction of Th2 cells (Le Gros et al., 1990). Treatment of L. major infected Balb/c mice with multiple doses of anti-IL-2 antibody mediated cure and was associated with increased IFN-γ and decreased IL-4 mRNA in regional lymph node T cells (Heinzel et al., 1993a). In tissue culture, recombinant IL-4 has been shown to inhibit priming of Th0 cells for development into IFN-γ secreting cells; this effect is diminished in the presence of IL-12 which promotes development of IFN-γ producing cells (Seder et al., 1992 and 1993; Hsieh et al. 1992). Similarly, treatment of resistant mice with recombinant IL-4 at the time of L. major infection blocked the induction of IFN-γ producing Th1 cells and led to induction of a Th2 response and exacerbation of disease (Chatelain et al., 1992).

Furthermore, IL-4 transgenic mice were shown to be susceptible to L. major infection whereas the parent strain was resistant (Leal et al., 1993).

Antigen presenting cells may also be involved in the differential induction of Th1 versus Th2 responses. Macrophages and B cells from resistant and susceptible mice are reported to differentially activate Th1 and Th2 subsets, respectively (Rossi-Bergmann et al, 1993). Macrophages were shown to stimulate secretion of IFN-γ and IL-2 by syngeneic Leishmania-specific T cells while B cells were shown to stimulate IL-4 secretion. Furthermore, macrophages from resistant mice stimulated a larger Th1 response than macrophages from susceptible mice. Conversely, B cells from susceptible mice stimulated a much greater Th2 response than B cells from resistant mice. Controls in which T cells from F1 hybrids were stimulated with parental macrophages or B cells gave similar results suggesting that the genetic differences in lymphokine secretion were primarily a function of the antigen presenting cells, not the T cells. These results are consistent with the finding that Balb/C mice are rendered resistant to L. major by depletion of B cells by anti-IgM treatment at birth whereas naturally resistant C3H mice are
unaffected (Sacks et al., 1984). However, Rossi-Bergmann et al. (1993) examined only peritoneal and splenic macrophages and not Langerhans cells which are thought to be important in antigen presentation for initiation of the primary cellular immune response. Langerhans cells have been reported to phagocytose L. major in the skin and, unlike infected macrophages, appear to migrate to the draining lymph nodes and stimulate naive T cells (Will et al., 1992; Blank et al., 1993; Moll et al., 1993).

A Th1/Th2 dichotomy is also thought to mediate either cure or disease progression in human leishmaniasis although a less polarized profile is observed relative to the L. major murine model. Leishmania-reactive CD4+ T cell clones with Th0, Th1 and Th2 profiles were isolated from patients who had recovered from VL following drug therapy (Kemp et al., 1993). Of 17 clones isolated, 8 had a Th1 profile secreting IFN-γ but not IL-4, 2 had a Th2 profile secreting IL-4 but not IFN-γ, and 7 had a Th0 profile secreting both IL-4 and IFN-γ. There is also evidence that different Leishmania antigens stimulate different Th subsets and that the response to a given antigen may be different between disease types. LPG-associated protein was shown to stimulate a Th1 type response in VL patients cured by drug therapy whereas GP63 stimulated a Th2 response (Kurtzhals et al., 1994). In contrast, GP63 stimulated a Th1 type response in T cell lines from patients with CL caused by L. amazonensis (Russo et al., 1991). These T cell lines were shown to contain both CD4+ and CD8+ T cells and secreted IL-2 and IFN-γ but not IL-4. Lymphokine mRNAs resembling a Th1 response (IL-2, IFN-γ) were also detected in human CL lesions which spontaneously heal (Pirmez et al., 1993). Conversely, lymphokine mRNAs resembling a Th2 response (IL-4, IL-10) were detected in MCL lesions which are chronic and progressive.

(2) immune effector mechanisms

The protective effect of the Th1 response in murine Leishmania infections appears to be mediated by induction of a toxic nitric oxide (NO) response in infected macrophages;
a respiratory burst may also be activated but is not required for killing of intracellular amastigotes (Scott et al., 1985; Assreuy et al., 1994). An inducible nitric oxide synthetase (iNOS) oxidates L-arginine to produce citrulline and NO which is spontaneously converted to NO₂⁻ and NO₃⁻ (Marletta et al., 1988; Hevel et al., 1991; Stuehr et al., 1991; Xie et al., 1992; Lyons et al., 1992). NO inhibits mitochondrial respiration by nitrosylation of Fe-S prosthetic groups necessary to the function of the electron transport chain (Drapier and Hibbs, 1988; Stuehr and Nathan, 1989; Lancaster and Hibbs, 1990; Pellat et al., 1990; Drapier et al., 1991). In addition, NO may participate in the formation of toxic hydroxyl radicals (Beckman et al., 1990). L. major promastigotes are killed by NO in vitro and the leishmanicidal activity of activated macrophages is markedly decreased by a competitive inhibitor of iNOS, L-N-monomethyl arginine (L-NMMA) (Liew et al., 1990a).

Furthermore, injection of L-NMMA into footpad lesions of L. major exacerbated infection and increased parasite load by 10,000 fold.

IFN-γ has been shown to stimulate production of TNF-α by macrophages infected with L. major amastigotes and these two cytokines synergistically activate NO production and concomitant killing of intracellular amastigotes (Liew et al., 1990c; Stenger et al., 1991). IL-2 in combination with IFN-γ and TNF-α is reported to further enhance induction of iNOS (Deng et al., 1993). In contrast, TNF-α in combination with IL-4 does not activate killing (Bogdan et al., 1990) and IL-10, which is produced by Th2 cells, appears to block NO production by inhibiting TNF-α production by macrophages (Oswald et al., 1992). These results are consistent with the protective effect of recombinant TNF-α and the exacerbative effects of anti-TNF-α antibodies demonstrated in vivo (Liew et al., 1990b). Recently, macrophages in cutaneous lesions and draining lymph nodes of resistant C57BL/6 mice were shown to contain high levels of iNOS protein, relative to levels in susceptible Balb/C mice, and iNOS was detected much earlier in the infection (Stenger et al., 1994). Furthermore, lesions of Balb/c mice displayed high levels of TGF-β which has been shown to inhibit iNOS in vitro (Ding et al., 1990).
Numerous studies with human macrophages have failed to demonstrate expression of human iNOS (Chartrain et al., 1994) or production of NO under conditions shown to activate murine macrophage iNOS expression (Schneemann et al., 1993; Roland et al., 1994; Chesrown et al., 1994; Bermudez et al., 1993). Therefore activation of the macrophage respiratory burst may be of greater importance in humans for killing of intracellular amastigotes. In contradiction with these recent studies, Munoz-Fernandez et al. (1992) reported TNF-α/IFN-γ mediated induction of NO production in human macrophages and concomitant activation of killing of intracellular Trypanosoma cruzi. However, neither the statistical significance of the data nor the number of replicates was indicated in this report. Furthermore, NO production was inhibited only two fold by addition of 100 μM L-NMMA whereas Liew et al. (1990c) reported complete inhibition of murine macrophage NO production at 50 μM L-NMMA and observed a 2 fold inhibition with 0.5 μM L-NMMA. Moreover, Munoz-Fernandez et al. (1992) did not demonstrate a direct effect of L-NMMA on inhibition of macrophage killing of intracellular amastigotes and did not use D-NMMA as a negative control. Thus, there is no compelling evidence for NO production in human macrophages.

(3) innate resistance in murine leishmaniasis.

Innate resistance and susceptibility of mice to L. donovani is controlled by a single gene (referred to as Lsh or Ity or Bcg) also thought to control the early response to Salmonella typhimurium and Mycobacterium bovis, both of which infect macrophages (Gros et al., 1981; Skamene et al., 1982). It has been hypothesized that the Lsh gene regulates macrophage priming for antimicrobial activity via TNF-α-dependent induction of iNOS although expression of the Lsh gene appears to be restricted to particular macrophage populations. Differences in the anti-leishmanial activity of macrophages from Lsh^F and Lsh^S mice have been reported for bone marrow-derived macrophages and resident macrophages from liver and lung but not for peritoneal or splenic macrophages (Crocker
et al, 1984 and 1987; Roach et al., 1991; Zwilling and Hilburger, 1994). For bone marrow derived macrophages the increased killing ability of IFN-γ/ lipopolysaccharide activated LshF macrophages relative to LshS macrophages was correlated with TNF-α and NO release and was inhibited by either anti-TNF-α antibody or L-NMMA (Roach et al., 1991). *L. major*, which is normally not influenced by the Lsh gene, has been shown to preferentially infect infiltrating monocytes in the liver whereas *L. donovani* infects the resident liver macrophages functionally demonstrated to express the Lsh gene (Davies et al, 1988). Moreover, the Lsh gene was shown to exert some influence over *L. major* infection in the absence of infiltrating monocytes eliminated by prior irradiation. A candidate Lsh gene termed Nramp has recently been cloned and sequenced and is predicted to encode a macrophage transmembrane protein with three potential protein kinase C phosphorylation sites and with sequence similarity to nitrate transporters (Vidal et al., 1993; Barton et al., 1994). It has been hypothesized that Nramp might be involved in inducible delivery of nitrates to the phagolysosome compartment wherein the acid environment could mediate conversion of nitrate to NO. However this suggestion does not account for the apparent effects of Lsh on iNOS expression and these predictions have yet to be verified by functional studies of the recombinant protein. Moreover, the Nramp gene has not yet been shown to confer the LshF phenotype.

### E. Evasion of Non-specific Host Defenses

In order to survive in the mammalian host, *Leishmania* has evolved complex mechanisms to subvert and evade host immune responses. A specific primary immune response takes weeks to fully develop; therefore evasion of nonspecific immune defenses is critical to establishment of infection. There is considerable evidence that surface
components of *Leishmania* promastigotes play critical roles in serum resistance and in the initiation and establishment of macrophage infection.

(1) **complement fixation and serum resistance**

All developmental stages of *Leishmania* have been shown to activate complement by the alternative pathway (Mosser and Edelson, 1984; Mosser *et al.*, 1985; Blackwell *et al.*, 1985; Russell, 1987) but there is species variation in the predominate form of C3 bound on the cell surface. Although both C3b and iC3b can be detected, the majority of the bound C3 was C3b on *L. major* and *L. mexicana* promastigotes and iC3b on *L. donovani* (Mosser and Edelson, 1985; Puentes *et al.*, 1988 and 1989; Russell, 1987). GP63, in *L. mexicana* promastigotes, and LPG, in *L. major* promastigotes, have been implicated as the major acceptor sites for C3b deposition. C3b was immunoprecipitated with anti-GP63 antibodies but not anti-LPG antibodies from *L. mexicana* promastigotes incubated with radioiodinated C3 (Russell, 1987). Furthermore, liposomes containing *L. mexicana* GP63, but not LPG, were shown to bind C3 in the presence of complement factors B and D. In contrast, the majority of radioiodinated C3 bound on the surface of *L. major* promastigotes was immunoprecipitated with anti-LPG antibodies (Puentes *et al.*, 1988).

Despite complement activation, metacyclic promastigotes are resistant to complement mediated cell lysis whereas procyclic promastigotes are not (Puentes *et al.*, 1988). During metacyclogenesis, LPG side chains are modified and the molecule is elongated by an increase in the number of repeating disaccharide units (Sacks *et al.*, 1990; McConville *et al.*, 1992, reviewed by Sacks *et al.*, 1994). This elongation thickens the glycocalyx (Pimenta *et al.*, 1991) and appears to inhibit complement mediated cell lysis of *L. major* promastigotes by preventing access of the C5b-9 membrane attack complex to the promastigote membrane (Puentes *et al.*, 1990).

It has also been suggested that GP63 may cleave C3b to inactive peptides thereby down regulating complement activation. Release of proteolytic fragments of C3b from the
surface of *L. donovani* has been reported (Puentes *et al*., 1989) and purified *L. mexicana* GP63 has been shown to cleave C3 (Chaudhuri and Chang, 1988). However, cleavage of C3b by GP63 on the promastigote surface has not been directly demonstrated. Chaudhuri and Chang (1988) also suggested that GP63 may cleave C3 to C3b at the promastigote surface to initiate complement activation. However, hydrolysis of C3 to a C3b-like molecule, C3(H2O), occurs spontaneously at a low level in the circulation to form fluid phase C3 convertase, C3(H2O)Bb, and thereby initiate complement fixation when deposited on the cell surface of an invasive micro-organism (Pangburn *et al*., 1980, 1981, 1983). Therefore, activation of the alternative pathway may be enhanced by proteolytic cleavage of C3 by the parasite but would not be dependent on it.

(2) promastigote - macrophage interactions: attachment and phagocytosis

In addition to evasion of complement-mediated lysis, *Leishmania* metacyclic promastigotes have been shown to exploit complement mediated phagocytosis to gain entry into host macrophages and thereby avoid activation of an oxidative burst. Both CR1, the receptor for C3b, and CR3, the receptor for iC3b, have been implicated in serum opsonized macrophage binding (Mosser and Edelson, 1985; Blackwell *et al*., 1985, Da Silva et al, 1989). As discussed previously, both LPG and GP63 have been reported to bind C3b and iC3b. Although there is evidence that GP63 and LPG may bind CR3 directly (Russell and Wright, 1988; Talamas and Russell, 1989), it appears that in the presence of serum, binding of *Leishmania* to macrophage complement receptors is mediated by C3b and/or iC3b depending on the species of *Leishmania* involved. Complement mediated phagocytosis via CR1 and CR3 avoids activation of a respiratory burst (Berton and Gordon, 1983, Wright and Silverstein, 1983) and therefore may promote intracellular survival of *Leishmania* metacyclic promastigotes. In contrast, phagocytosis mediated by both CR3 and the mannose-fucose receptor in the absence of serum, appears to activate a respiratory burst and effect killing of promastigotes (Blackwell *et al*., 1985). Several
weeks post-infection as specific anti-parasite IgG antibodies appear (Escobar, 1992), antibody mediated phagocytosis of amastigotes via macrophage FcγR may occur (Guy and Belosevic, 1993) and trigger an oxidative burst (Wright and Silverstein, 1983), thereby promoting killing of the intracellular amastigotes. Similarly, it has been suggested that IgG may promote non-permissive phagocytosis of promastigotes in immune individuals re-exposed to Leishmania (Mosser and Rosenthal, 1993). However, as previously discussed LPG has been shown to block binding of human immune sera to L. donovani (Karp et al, 1991).

Studies on Leishmania-macrophage interactions have been complicated by several factors: (1) differences between procyclic and metacyclic promastigotes; (2) differences among Leishmania species; (3) differences between human versus murine macrophages; and (4) the presence or absence of serum. Moreover, studies done in the absence of serum may be confounded by local secretion of complement components by macrophages (Whaley, 1980). For example, studies examining the serum-independent binding of beads coated with either GP63 or LPG to murine macrophages indicated direct binding of these molecules to CR3 (Russell and Wright, 1988; Talamas and Russell, 1989). Furthermore, the conserved sequence SRYD at position 252-255 in L. major GP63 is thought to mimic the RGDS sequence of fibronectin and mediate direct binding to CR3 at the RGD acceptor site shown to bind iC3b (Russell and Wright, 1988; Russell et al., 1989; Soteriadou et al, 1992; Wright et al., 1987). However, Mosser et al. demonstrated that binding of stationary phase L. major, L. donovani, and L. amazonensis promastigotes required complement for binding to either CR3 coated plates or to COS cells expressing recombinant CR3 (Mosser et al., 1992). Studies of amastigotes and metacyclic promastigotes, in the presence of serum, are likely to be more relevant to the mechanisms in vivo. The presence of serum has been shown to enhance binding of L. enrietti, L. major, L. donovani and L. tropica promastigotes to macrophages although the level of enhancement varies among species (Mosser and Edelson, 1984; Mosser and Rosenthal, 1993; Blackwell et al., 1985).
Anti-CR3 antibodies have been reported to inhibit the binding of *L. major* stationary phase promastigotes to murine macrophages by 50 - 60% in the presence and absence of serum (Mosser and Edelson, 1984 and 1985). Similarly, anti-CR3 and anti-C3 antibodies have been shown to inhibit binding of *L. donovani* promastigotes to murine macrophages in the presence of serum (Blackwell *et al.*, 1985). Cooper *et al.* demonstrated that the binding of *L. major* and *L. donovani* procyclic promastigotes to murine macrophages was inhibited by both mAb OKM10, directed to the iC3b binding site of CR3, and mAb OKM1, directed to a lectin-like site on CR3 (Ross *et al.*, 1985; Cooper *et al.*, 1988). In contrast, the binding of metacyclic promastigotes (metacyclics) was not inhibited by mAb OKM10 directed to the iC3b site. Moreover, treatment with sodium salicyl hydroxamate, which inhibits covalent ester linkage of C3b to the parasite surface, inhibited procyclic promastigotes (procyclics) but not metacyclics. It has also been shown that binding of *L. major* promastigotes to murine macrophages is significantly inhibited in the presence of anti-LPG antibodies (Handman and Goding, 1985; Kelleher *et al.*, 1992). Therefore, it was suggested that binding of procyclics to CR3 is iC3b mediated whereas binding of metacyclics to CR3 involves direct lectin-like binding to CR3 by LPG. Binding studies using beads coated with LPG purified from *L. mexicana* stationary phase promastigotes are consistent with this hypothesis; the reported serum independent binding of LPG-coated beads to human macrophages was also inhibited by mAb OKM1 (Talamas-Rohana *et al.*, 1990). The developmental modification of LPG during metacyclogenesis may account for the reported differences between procyclics and metacyclics.

In contrast to these studies, the binding of *L. major* PNA+ procyclics to human macrophages is reportedly mediated by lectin-like binding to CR3 whereas binding of PNA- metacyclic promastigotes is mediated by CR1 (Da Silva *et al.*, 1989). This is consistent with the observation that the majority of C3 bound on *L. major* promastigotes is C3b. Furthermore, serum complement opsonization was shown to be required for significant binding and phagocytosis of metacyclics. Da Silva *et al.* (1989) suggested that
C3b-mediated binding to CR1 favours phagocytosis in the absence of a respiratory burst as observed for metacyclics. Moreover, it is suggested that the strong respiratory burst observed upon phagocytosis of procyclic promastigotes is triggered by ligation of the lectin site of CR3. The studies by Cooper et al. (1988) and by Talamas-Rohana et al. (1990) used unpurified stationary phase cultures as metacyclics and did not investigate a role for CR1. Contamination with procyclic promastigotes could possibly account for the discrepancy between their observations and the findings of Da Silva et al. (1989). Differences between murine and human macrophages and between the species or strain of *Leishmania* studied may also be important.

In most of the studies described above, blocking of a single complement receptor inhibited binding of promastigotes to macrophages by only 50 - 80%. Furthermore, whereas beads coated with both LPG and GP63 were phagocytosed by murine macrophages, beads coated with either molecule alone were not, despite significant binding (Russell and Wright, 1988). Thus, numerous promastigote-macrophage interactions may facilitate binding and promote the specific interactions among GP63, LPG, complement, CR3, and CR1 necessary to trigger phagocytosis. Both the macrophage mannose-fucose receptor and the macrophage receptor for advanced glycosylation endproducts have been implicated in attachment of *Leishmania* to macrophages (Blackwell et al., 1985; Wilson and Pearson, 1986 and 1988; Mosser et al., 1987). Heparin binding by *L. amazonensis* amastigotes has also been shown to mediate adhesion to macrophages and other cells expressing heparan sulfate proteoglycans (Love et al., 1993). Similarly, fibronectin has been implicated in mediating attachment of promastigotes to macrophages (Wyler et al., 1985).

(3) survival within the macrophage phagolysosomal compartment

Both LPG and GP63 have been implicated in promoting survival of the parasite within the macrophage phagolysosomal compartment by circumventing the toxic
respiratory burst and avoiding degradation by macrophage lysosomal enzymes. Reduced expression of either GP63 or LPG has been correlated with reduced virulence in strains of
*L. major*, *L. donovani* and *L. mexicana* (Shankar et al., 1993; Chaudhuri et al., 1988; McNeely and Turco, 1990).

As discussed previously, LPG and GP63 promote complement mediated phagocytosis by receptors CR3 and CR1 which may avoid activation of a respiratory burst. Moreover, infected macrophages have been shown to be resistant to activation of a respiratory burst by LPS (Buchmuller-Rouiller and Mauel, 1987). Appropriate receptor-ligand interactions on the macrophage cell surface normally lead to activation of phosphokinase C and its translocation to the plasma membrane which triggers the respiratory burst by activation of NADPH-oxidase (Baggiolini and Wymann, 1990). Superoxide (O$_2^-$), which is spontaneously converted to H$_2$O$_2$, is released at the cell surface and into phagocytic vesicles by activated NADPH-oxidase. Purified LPG has been shown to inhibit phosphokinase C and to scavenge O$_2^-$ metabolites (Chan et al., 1989; McNeely and Turco, 1987 and 1990). Furthermore, phosphokinase C-mediated phosphorylation was inhibited in macrophages pretreated with purified LPG or infected with *L. donovani* promastigotes (Descoteaux and Turco, 1993). Following phagocytosis of *Leishmania* promastigotes, LPG epitopes can be detected on the macrophage cell surface and are maximally expressed during the first 48 hours post-infection during which promastigotes are converted to amastigotes (Tolson et al., 1990). LPG expression is substantially reduced on amastigotes (Bahr et al., 1993) and by 5 or 6 days post-infection, LPG is no longer detectable on the macrophage cell surface. Therefore, the protective effects of LPG may be limited to the critical early stages of promastigote infection.

GP63 may have a role in protection from lysosomal enzymes. Purified GP63 from *L. mexicana* promastigotes has been reported to degrade lysosomal proteins at acidic pH *in vitro* (Chaudhuri and Chang, 1988). Moreover, radioiodinated BSA in liposomes coated with native GP63 is protected from phagolysosomal degradation in macrophages
(Chaudhuri et al., 1989). In contrast, liposomes coated with denatured GP63 were not protective. Therefore, it was suggested that GP63 may protect Leishmania by proteolytic degradation of lysosomal enzymes. The demonstration that red blood cells coated with LPG resist lysis by macrophages suggests that LPG may also have a role in protecting promastigotes from lysosomal degradation (Eilam et al., 1985).

The protective roles of LPG and GP63 described above, may function principally in the establishment of infection by protecting the more vulnerable promastigote stage of the parasite. Molecules secreted or expressed on the surface of the amastigote stage may be important for persistence within the host macrophage and establishment of chronic infection. In comparison to promastigotes, L. donovani amastigotes have been shown to be more resistant to hydrogen peroxide in vitro and to survive in human monocytes despite eliciting an oxidative burst (Pearson et al., 1983; Murray, 1982). In comparison to promastigotes, L. donovani Amastigote lysates have high activity of glutathione peroxidase, catalase and superoxide dismutase which are thought to mediate H2O2 resistance. (Murray, 1981 and 1982). Compared to promastigotes, L. mexicana amastigotes have been reported to have increased levels of acid phosphatase which may inhibit macrophage activation by dephosphorylating critical substrates of phosphokinase C (Remaly et al., 1985; Menz et al., 1991).

In addition to the defense mechanisms described above, there is also evidence that dermal macrophages, the target of promastigote infection in vivo, have reduced leishmanicidal capacity compared to monocytes. Locksley et al. (1988) demonstrated that L. major promastigote infection of primate dermal macrophages was not associated with a respiratory burst and led to successful differentiation to amastigotes and persistent infection. In contrast, survival and replication of parasites in infected murine peritoneal macrophages, human monocytes and primate monocytes was markedly reduced and correlated with a strong respiratory burst. Similarly L. donovani promastigotes were readily killed by human monocytes whereas amastigotes are not (Pearson et al., 1983).
F. The Leishmania Surface Proteinase GP63 and its Similarity to Mammalian Matrix Metalloproteinases

(1) GP63

GP63 appears to be encoded by a divergent family of tandemly linked genes encoding different isoforms of GP63 that are developmentally regulated (Medina-Acosta et al., 1993; Ramamoorthy et al., 1992; Button and McMaster, 1988; Button et al., 1989; Voth and McMaster, manuscript in preparation). All Leishmania species examined express cell surface GPI-linked GP63 in the promastigote life stage and strains of L. major (Frommel et al., 1990; Pimenta et al., 1991) and L. mexicana (Medina-Acosta et al., 1989) have been shown to express an amastigote form of GP63. Some Leishmania strains, however, may not express amastigote GP63 (Schneider et al., 1992). Furthermore, recent studies have reported that L. mexicana amastigotes predominantly express a soluble form of GP63, with an acidic pH optimum, localizing to the amastigote lysosomal compartment (Ilg et al., 1993). The differences in GP63 gene expression observed for distinct developmental forms of Leishmania may reflect differences in the environmental conditions encountered by procyclics, metacyclics and amastigotes. Apart from the demonstrated role of GP63 in complement mediated phagocytosis and its suggested role in protection from lysosomal enzymes, little is known about the function of GP63 proteinase activity in the life cycle of the parasite.

L. major GP63 has been predicted to be synthesized as a 602 amino acid precursor protein (Button and McMaster, 1988) containing an NH₂-terminal leader sequence of 39 amino acids, a putative regulatory pro region of 61 amino acids, the mature protein of 477 amino acids, and a COOH-terminal GPI attachment signal sequence of 25 amino acids (Figure 4). Promastigote GP63 has been reported to be a neutral site specific endopeptidase cleaving a variety of substrates such as casein, gelatin, albumin, hemoglobin
Figure 4  Schematic diagram of predicted post-translational processing of Leishmania GP63

*L. major* GP63 has been predicted to be synthesized as a 602 amino acid precursor protein (Button and McMaster, 1988) containing an NH2-terminal leader sequence of 39 amino acids (pre), a putative regulatory pro region of 61 amino acids (pro), the mature protein of 477 amino acids, and a COOH-terminal GPI attachment signal sequence of 25 amino acids (C). GP63 also contains a putative Zn$^{2+}$ binding active site and at least two sites for N-linked glycosylation (CHO = carbohydrate).
and fibrinogen (Bouvier et al., 1989 and 1990; Tzinia and Soteriadou, 1991 Chaudhuri and Chang, 1988). GP63 purified from L. major promastigotes has also been shown to contain an equal molar ratio of zinc and is inhibited by the zinc chelator 1,10-phenanthroline (Etges et al., 1986b; Chaudhuri and Chang, 1988; Etges et al., 1989; Bouvier et al., 1989). Like other metalloproteinases, GP63 cleaves peptides on the amino side of the recognition residue (Bouvier et al., 1990). Using defined peptide substrates, the specificity of GP63 was shown to be determined largely by the P1' site (Schecter and Berger, 1967) with a preference for hydrophobic amino acids although polar residues were also observed at the P1' site (Bouvier et al, 1990).

Based on the predicted protein sequence of the cloned L. major GP63 gene (Button and McMaster, 1988), GP63 contains a putative active site, HEMAH (Bouvier et al., 1989). This region is homologous to the HExxH consensus sequence of a group of well characterized zinc metalloproteinases referred to as the 'zincins' superfamily where the underlined Glu residue participates in the catalysis of the substrate peptide bond and the two His residues are involved in coordination of the active site zinc (Matthews et al., 1972a,b and 1988; Hangauer et al, 1984; Vallee and Auld, 1990; Jongeneel et al., 1989; McMaster et al., 1994; reviewed by Hooper, 1994). X-ray crystallography of a number of well characterized zincins has revealed a common fold in the active site domain of these proteinases and suggests the existence of two sub-families (reviewed by Hooper, 1994; Blundell, 1994; Bode et al., 1994). In the 'gluzincins' sub-family, typified by thermolysin, the active site zinc is tetrahedrally coordinated by the 2 His residues in the HExxH motif, a distal Glu residue, and a water molecule (reviewed by Hooper, 1994). In the 'metzincins' sub-family, which includes the astacins, snake venoms, and matrix metalloproteinases, the active site zinc is coordinated by the 3 His residues in the extended motif HExxHxxGxxH and by a water molecule (Bode et al., 1993; Hooper, 1994). In the case of astacin, a distal Tyr residue constitutes a fifth zinc ligand (Gomis-Ruth et al.,
The metzincins also share a common methionine-containing turn which forms a hydrophobic base for the active site residues (Bode et al., 1993).

Site directed mutagenesis of the proposed GP63 catalytic Glu-265 to Asp-265 resulted in expression of rGP63 with no metalloproteinase activity, implying that GP63 Glu-265 was essential for proteinase activity (McMaster et al., 1994; Macdonald et al., submitted). This conservative mutation would result in a decrease by one carbon atom of the side chain of this residue and would not likely disrupt the tertiary structure of the GP63 active site. Thus, the mechanism of GP63 metalloproteinase activity is predicted to be homologous to that of thermolysin (Hangauer et al., 1984; Matthews et al., 1988). GP63 also exhibits several characteristics of the matrix metalloproteinases (MMPs) which are grouped in the metzincins sub-family (Nagase et al., 1992; Bode et al., 1993). Similar to MMPs, GP63 is expressed at the cell surface, degrades extracellular matrix components such as fibrinogen (Bouvier et al., 1989), and is inhibited by α2-macroglobulin (Heumann et al., 1989).

(2) Matrix metalloproteinases and the extracellular matrix

The MMP family includes a group of nine or more mammalian zinc metalloproteinases with overlapping substrate specificities (Table 1). Sequence comparison of genes encoding human and animal MMPs revealed extensive sequence similarities and a highly conserved domain structure among the MMPs suggesting that individual enzymes consist of different combinations of 6 domains (Figure 5). Collectively, the MMPs degrade most of the components of the extracellular matrix.

The extracellular matrix (ECM) is a complex mixture of proteinaceous fibres such as collagen and elastin embedded within a gel like "ground substance" consisting mainly of glycosaminoglycans and proteoglycans (Alberts et al., 1994). The aqueous phase of the ground substance permits diffusion of nutrients, metabolites, and cell-signalling molecules such as hormones between blood and the tissue cells. Collagen fibrils provide tensile
Table 1  The matrix metalloproteinase family*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>MMP#</th>
<th>Mr.</th>
<th>Extracellular matrix substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast-type collagenase</td>
<td>FIB-CL</td>
<td>MMP-1</td>
<td>57,000/52,000</td>
<td>Collagen I, II, III, (III&gt;&gt;I), VII, VIII, X; gelatin; PG core protein</td>
</tr>
<tr>
<td>PMN-type collagenase</td>
<td>PMN-CL</td>
<td>MMP-8</td>
<td>75,000</td>
<td>Same as FIB-CL (I&gt;&gt;III)</td>
</tr>
<tr>
<td>Stromelysin-1</td>
<td>SL-1</td>
<td>MMP-3</td>
<td>60,000/55,000</td>
<td>PG core protein; fibronectin; laminin; collagen IV, V, IX, X; elastin; proCL</td>
</tr>
<tr>
<td>Stromelysin-2</td>
<td>SL-2</td>
<td>MMP-10</td>
<td>60,000/55,000</td>
<td>Same as SL-1</td>
</tr>
<tr>
<td>Stromelysin-3</td>
<td>SL-3</td>
<td>MMP-11</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Macrophage metalloelastase</td>
<td>MME</td>
<td>?</td>
<td>53,000</td>
<td>Elastin</td>
</tr>
<tr>
<td>M, 72K gelatinase type IV collagenase</td>
<td>M, 72K GL</td>
<td>MMP-2</td>
<td>72,000</td>
<td>Gelatin; collagen IV, V, VII, X, XI; elastin; fibronectin; PG core protein</td>
</tr>
<tr>
<td>M, 92K gelatinase type IV collagenase</td>
<td>M, 92K GL</td>
<td>MMP-9</td>
<td>92,000</td>
<td>Gelatin; collagen IV, V; elastin; PG core protein</td>
</tr>
<tr>
<td>Putative metalloproteinase-1</td>
<td>PUMP-1</td>
<td>MMP-7</td>
<td>28,000</td>
<td>Fibronectin, laminin, collagen IV, gelatin, proCL, PG core protein</td>
</tr>
</tbody>
</table>

Note: n.d.: not determined.


* this table was taken from Birkedal-Hansen et al, 1993
Figure 5  The domain structure of MMPs
The MMP prototype consists of 5 different domains: (1) a signal peptide of 17-29 aa (2) a 77-87 aa propeptide that constitutes the NH2-terminal domain of the secreted proMMP precursors, (3) a catalytic domain that contains the Zn\(^{2+}\) binding site, (4) a 5-50 aa proline-rich hinge region that marks the transition to the COOH-terminal domain, and (5) a 200 aa hemopexin or vitronectin-like COOH-terminal domain that appears to play a role in substrate specificity. MMP-7 lacks the fifth domain and MMP-2 and MMP-9 contain an additional fibronectin type II-like domain. This figure was taken from Birkedal-Hansen et al., 1993.
strength whereas elastin provides elasticity. Fibroblasts are largely responsible for synthesis of the ECM components and influence their organization within the matrix. Glycoproteins such as fibronectin are involved in anchoring fibroblasts and other resident cells within the ECM and a variety of adhesive molecules are thought to assist in cell migration through the matrix. In general, the ECM occupies the extracellular space within vertebrate tissues. In connective tissues, the ECM is generally much more abundant than the surrounding cells and determines the structure of the tissue. The amounts of connective tissue found in different organs varies greatly; in skin, connective tissue is a major component and constitutes the dermis. A specialized extracellular matrix termed basal lamina or basement membrane is produced by and underlies all epithelial cell sheets and separates the epidermis from the dermis in skin. The basal lamina is a thin meshlike sheet of mainly type IV collagen molecules associated with glycoproteins and proteoglycans that mediate attachment to the overlying basal epithelial cells and the underlying connective tissue.

Cell migration through the extracellular matrix is essential not only for development, inflammation, and wound repair, but is also necessary for tissue invasion by metastatic cells. Degradation of the ECM in connective tissues and basal laminae principally involves the serine proteases of the plasmin/plasminogen activator system and the MMPs although some cysteine proteases (e.g. cathepsin B) and endo- and exo-glycosidases also contribute (reviewed by Mignatti and Rifkin, 1993). Collectively, these enzymes are capable of degrading virtually all of the ECM components. Activation of the proenzyme plasminogen to plasmin is thought to be key in initiating the cascade. Plasmin, a ubiquitous plasma protein, is a broad-spectrum serine protease that in addition to its role in degrading fibrin blood clots also cleaves a number of ECM components and is thought to mediate proteolytic activation of a number of different proMMPs. Plasminogen activation is localized at the cell surface by urokinase type plasminogen activator (uPA) which is a receptor bound serine proteinase that activates plasminogen. uPA is secreted as a zymogen
(pro-uPA) and then bound by a cell surface receptor. Expression of the uPA receptor is modulated by several factors including cytokines. Activation of pro-uPA can be mediated by plasmin and cathepsin B; other unknown proteases may also be involved.

A number of proteins found in plasma and interstitial fluids have been shown to inhibit MMPs and thereby down-regulate degradation of the ECM. Mammalian α2-macroglobulin inactivates MMPs by forming covalent multimeric complexes. Proteolytic cleavage of a 'bait' region in the inhibitor induces a conformational change and hydrolysis of an internal thiol-ester bond, thus exposing a reactive glutamyl residue which reacts with a lysyl side chain on the MMP to form a covalent bond (Sottrup-Jensen et al., 1983 and 1989; Sottrup-Jensen, 1989). Tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and TIMP-2) form non-covalent bimolecular complexes with active MMPs and some proMMPs thereby inhibiting MMP activity and/or activation (DeClerck et al., 1991; Stetler-Stevenson et al., 1992; Stricklin and Welgus, 1983; reviewed by Denhardt et al., 1993).

The MMPs are synthesized as latent pro-enzymes requiring activation and processing for full proteinase activity (Grant et al., 1992; Birkedal-Hansen et al., 1993). Activation of MMPs is thought to occur by a mechanism termed the cysteine switch (Figure 6) (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). According to this model, latency is maintained by blockage of the active site by coordination of the active site zinc with a sulfhydryl group of a conserved Cys residue in the pro region. Conditions which promote dissociation of the Cys from the active site allow coordination of the zinc ion by H2O which is required for catalysis (Vallee and Auld, 1992). This leads to activation of the proenzyme and subsequent autolytic removal of the pro peptide. Mercurial compounds, which are thought to coordinate sulfhydryl groups, have been reported as potent activators of latent MMPs (Stricklin et al., 1983; Grant et al., 1987; Springman et al, 1990; Crabbe et al., 1992). It was recently hypothesized that rGP63, secreted in the baculovirus insect cell system, was expressed as a latent proteinase activated in vitro according to the cysteine switch pathway described for MMPs (Button et al., 1993).
Figure 6  The cysteine switch mechanism

The activation of latent matrix metalloproteinases by the cysteine switch mechanism is depicted schematically. In the latent form, the fourth coordination site of the active site zinc is bound by the sulfhydryl group of a cysteine in the pro region. Various conditions which disrupt the Cys-Zn$^{2+}$ interaction lead to activation of proteinase activity and subsequent autolytic removal of the pro peptide. In the active form, the fourth coordination site of the Zn$^{2+}$ is bound by a water molecule which is essential for catalysis. In this diagram Cys-73 refers to the position of the pro region Cys in human fibroblast procollagenase. This figure was taken from Springman et al, 1990.
G. Objectives of this study

There is considerable evidence that *Leishmania* cell surface molecules play critical roles in the initiation and establishment of macrophage infection, evasion of non-specific immune defenses, and in stimulation of a cellular and humoral immune response. The overall objectives of this study were to examine the activation mechanism of recombinant GP63 (rGP63) and to identify novel glycoproteins expressed on the surface of *L. mexicana* amastigotes.

(1) analysis of the activation mechanism and processing of GP63

GP63 has been shown to demonstrate a number of similarities to the MMP family. Previous studies demonstrated that rGP63 expressed by the baculovirus insect cell system was secreted as a glycosylated latent proteinase (rGP63s) that required activation with mercurial compounds for full proteinase activity (Button et al., 1993). It was therefore hypothesized that rGP63s may be activated according to the cysteine switch pathway described for activation of proMMPs. To extend these studies the activation of rGP63 was examined using both secreted and cell surface expression systems with the following objectives:

i) to determine if the activation of latent rGP63s is mediated by autolytic removal of the predicted pro peptide according to the cysteine switch mechanism;

ii) to determine if expression as an active proteinase is linked to cell surface expression.

Analysis of the activation mechanism of GP63 may provide important insights toward understanding the regulation of GP63 proteinase activity. Further establishing the functional similarity of GP63 to the family of MMPs may have also have important implications regarding the function of GP63 proteinase in the mammalian host.
(2) identification of novel amastigote glycoproteins

Relative to the promastigote, the amastigote cell surface remains relatively uncharacterized. Analysis of the amastigote cell surface may lead to a better understanding of persistence of the amastigote within the host macrophage and may also lead to identification of novel antigens important to the host immune response to Leishmania. Amastigote surface molecules could also be important targets for both chemotherapeutic strategies and vaccine development. The potential of glycoprotein purification to enrich for cell surface proteins was therefore exploited in a search to identify novel amastigote surface molecules. The specific objectives were as follows:

(i) to determine the suitability of in vitro amastigotes for characterization of proteins expressed in vivo;
(ii) to demonstrate the efficacy of lectin affinity chromatography to enrich for cell surface molecules;
(iii) to identify unique amastigote glycoproteins by NH2-terminal amino acid sequence analysis.

Determination of unique protein sequence(s) will enable isolation of the corresponding gene(s) and subsequent determination of the primary structure which may indicate potential function. Expression and study of the recombinant protein may further establish the putative function of the identified proteins. Ultimately, the generation and analysis of Leishmania deletion mutants may establish a function in vivo.
Chapter 2. Materials and Methods

A. Leishmania

(1) maintenance of Leishmania promastigotes in vitro

_L. major_ (NIH S strain) and _L. mexicana_ (WHO designation MNYC/B2/62/M379) promastigotes were maintained at 25°C in M199 media (Gibco BRL) containing 10% fetal calf serum (FCS) (Hyclone) and 40 mM HEPES (pH 7.3) as previously described (Button et al., 1989).

(2) maintenance of L. mexicana amastigotes in vitro

_L. mexicana_ promastigotes at a density of 5 X 10^6 per ml were incubated overnight at 32°C in M199 plus 10% FCS and then diluted 1:1 in UM54 media (pH 5.5) plus 20% FCS and incubated at 32°C (D. Russell, personal communication). The cultures were then maintained at 32°C at densities ranging from 5 x 10^5 cells per ml to 3 x 10^7 cells per ml by routine passage in UM54 plus 20% FCS. UM54 media consisted of M199 (Gibco, BRL) with the following supplements: 0.25% glucose, 0.5% trypticase, 25 mM HEPES, 5.14 mM glutamine, and 0.035% NaHCO3.

(3) isolation of L. mexicana lesion amastigotes

Abdominal lesions were dissected from Balb/C mice 6-8 weeks post-infection by subcutaneous injection with lesion _L. mexicana_ amastigotes. Lesions were minced with a scalpel and then disrupted by passage through a metal mesh. Cells were resuspended in sterile PBS and disrupted further by successive passage through 18g, 22g, and 26g needles, to rupture host macrophage cells and create a single cell suspension of amastigotes. The resulting suspension was then centrifuged at 1100 X g in a benchtop centrifuge (Beckman TH-4 rotor) and resuspended in PBS and partially purified either by
centrifugation in a 2-step percoll (Sigma) gradient (D. Russell, personal communication) or by passage through absorbent cotton wool. The percoll gradient was prepared by layering amastigotes, resuspended in 6 ml of 35% (vol/vol) percoll in PBS, over 6 ml of 100% percoll. The 2-step gradient was then centrifuged for 25 min at 600 X g in a benchtop centrifuge (Beckman TH-4 rotor). The amastigotes were isolated from the interphase and washed with PBS.

B. Preparation of Rabbit Antisera

(1) production and affinity purification of a rabbit antiserum (anti-prepro) raised against the GP63 prepro peptide

A recombinant peptide corresponding to the 100 amino acid predicted prepro peptide of *L. major* GP63 was produced in *E.coli* and purified as previously described (Button et al., 1991). A rabbit antiserum was prepared by immunization of a rabbit with three successive injections, at 2 - 3 week intervals, of 100-125 µg purified recombinant prepro peptide in RIBI adjuvant. The immune serum (5 ml) was affinity purified by passage over a prepro peptide affinity column. Purified prepro peptide was dialyzed against 10 mM HEPES, pH 7.5, and coupled to 1 ml Affi-prep 10 beads (BioRAD) in 10 mM HEPES, pH 7.0, according to the manufacturer's protocol. Immune rabbit serum, diluted 1:1 in TBS, was applied to the prepro affinity column. The column was washed with 50 ml TBS and 1 ml fractions eluted with 0.15M NaCl, 0.05 M diethylamine, pH 11.5. The first two fractions were pooled and contained approximately 950 µg of antibody as determined by A280.
(2) production of an anti-amastigote rabbit serum and absorption with promastigotes

A rabbit antiserum was prepared by immunization of a rabbit with three successive injections of $10^6$ - $10^8$ L. mexicana lesion amastigotes in RIBI adjuvant followed by a fourth injection of amastigotes in complete Freunds adjuvant. For each injection fresh amastigotes were isolated from pooled lesions from 5 - 10 mice and purified on a percoll gradient.

The immune serum was absorbed with varying numbers of L. mexicana promastigotes. Promastigotes ($10^7$, $10^8$ and $10^9$) were pelleted by centrifugation, resuspended in 200 μl aliquots of the immune serum diluted 1/500 in DMEM+10%FCS, and incubated with gentle mixing for 1.5 hr at 4°C. The suspensions were then centrifuged for 7 min at 4000 X g in an eppendorf microfuge and the supernatants collected and tested by flow cytometry for reactivity with in vitro amastigotes and promastigotes.

C. Biotinylation of Amastigotes and Promastigotes

Approximately $10^9$ amastigotes or promastigotes were obtained from 100 ml of cell culture. The cells were washed four times with 1 volume of PBS and pelleted by centrifugation at 3200 X g for 10 min in a benchtop centrifuge. The washed cells were resuspended at 5 X $10^8$ cells per ml in PBS (i.e. 2 ml) containing 165 μg/ml BiotinXNHS (Calbiochem) and 5% DMSO, and then incubated for 30 min at 4°C with gentle agitation. 1 M Tris-HCl, pH 6.8 was then added to a final concentration of 50 mM and the cells were divided into two microfuge tubes and pelleted by centrifugation at 5000 x g in a microcentrifuge for 5 min at 4°C. The cells were then washed three times with 1.5 ml of TBS, pH 7.5. The combined cells were resuspended in 1 ml of lysis buffer (2% Zwittergent 3-14 (Calbiochem), 1 mM PMSF, 10 μM E64 in TBS pH 7.5) and incubated
for 30 min at 4°C with gentle agitation. The lysate was then centrifuged at 16 000 X g in a microcentrifuge for 15 min at 4°C and the supernatent stored at -20°C.

**D. Protein Purification**

(1) affinity purification of rGP63s

A mouse monoclonal antibody was prepared (R.P. Beecroft) as previously described (Pearson et al., 1980; Beecroft et al., 1993) following immunization of a Balb/c mouse with 25 μg purified *L. major* rGP63 synthesized by the baculovirus expression system (Button et al., 1993). This IgG₂a monoclonal antibody (referred to as #96) reacts with cell surface GP63 on *L. major* promastigotes but not with other species of *Leishmania*, as determined by flow cytometry analysis. Protein A purified #96 IgG (24 mg) was coupled to 5 ml Affi-prep 10 beads (BioRAD) in 10 mM HEPES (sodium salt) buffer, pH 7.0 according to the manufacturer's procedures. Recombinant GP63 (rGP63s), expressed by the baculovirus insect cell system using the *L. major* GP63 clone AcPP63.3 (Button et al., 1993) was produced in High Five cells using serum free medium (Invitrogen, San Diego, CA). Supernatant was centrifuged at 40,000 rpm for 1 hr at 4°C and 200 ml applied to the monoclonal #96 affinity column. The column was washed with 120 ml TBS and 1 ml fractions eluted with 0.15 M NaCl, 0.05 M diethylamine-HCl, pH 11.5 followed by neutralization with 1 M Tris-HCl, pH 8.0 as described (McMaster, 1984). Fractions containing rGP63s were pooled, dialyzed against TBS, aliquoted and stored at -20°C.
(2) glycoprotein purification using concanavalin A and lentil lectin affinity chromatography

Glycoproteins were purified from *L. mexicana* in vitro amastigote lysates by passage over either a concanavalin A (conA) sepharose column (Sigma) or a lentil lectin sepharose column (Sigma). The conA column was prepared by washing 3 ml of beads with 50 ml 0.2% Zwittergent 3-12 or 3-14, 0.5 M NaCl, 100 mM Tris pH 7.5, followed by 50 ml 0.2% Zwittergent 3-12 or 3-14 in TBS, pH 7.5 and then another 50 ml of the former. Approximately 10^10 amastigotes obtained from 1 - 2 litres of culture were washed 3 times with 1 volume of TBS. Cells were pelleted by centrifugation at 3840 X g for 10 min at 4°C (Beckman JA-14 rotor). Cells were resuspended at 10^9 cells per ml in lysis buffer (2% Zwittergent 3-14 or 3-12, 1 mM CaCl2, 1 mM MnCl2, 1 mM PMSF, 10 µM E64, in TBS pH 7.5) and incubated for 40 min at 4°C with gentle agitation. The lysate was centrifuged at 12,100 X g for 10 min at 4°C (Beckman JA-20 rotor) to pellet nuclei and cellular debris. The supernatant was then further centrifuged at 180,000 X g for 1 hour at 4°C in an ultracentrifuge (Beckman 70Ti rotor). The final supernatant was purified over a 3 ml conA sepharose column. The supernatant was applied to the column three times and allowed to flow through by gravity. The column was then washed with 50 ml 0.2% Zwittergent 3-12 or 3-14, 0.5 M NaCl, 100 mM Tris pH 7.5, followed by 50 ml 0.2% Zwittergent 3-12 or 3-14 in TBS, pH 7.5. The column was eluted in 10 ml fractions with 0.4 M alpha-methyl, alpha-D-glucopyranoside, 0.2% Zwittergent in TBS. Pooled eluates were concentrated by ultrafiltration and dialyzed extensively. Samples prepared with Zwittergent 3-14 were dialyzed against 4 L TBS whereas samples prepared with Zwittergent 3-12 were dialyzed against 4 L 0.01% Zwittergent 3-12 in TBS.

A lentil lectin sepharose column was prepared by washing 3 ml of beads with 50 ml of 0.2% sodium deoxycholate in 10 mM Tris pH 8.0, followed by 50 ml of 0.2% Zwittergent 3-14 in TBS, pH 7.5. An amastigote lysate was prepared and passed over the column as above. The column was washed with 40 ml of 0.2% Zwittergent 3-14 in TBS,
10 ml of 0.2% Zwittergent 3-14 in 10 mM Tris pH 8.0, and then 50 ml of 0.5% sodium deoxycholate in 10 mM Tris pH 8.0. The column was eluted with 0.4 M alpha-methyl, alpha-D-glucopyranoside, 0.5% sodium deoxycholate in TBS.

Lysates from biotinylated amastigotes were purified as above but on a smaller scale using only $10^9$ cells and a 0.5 ml column.

(3) Fractionation of purified glycoproteins by differential EtOH precipitation

The conA glycoprotein eluate and the lentil lectin glycoprotein eluate were each further fractionated by differential precipitation in EtOH. Pooled glycoprotein eluates, prepared as described in section D(2) above, were incubated for 48 hours at -20°C in a final concentration of 77% EtOH and then centrifuged at 16,000 X g in a microcentrifuge for 30 min at 4°C. The pellet was washed with 0.5 vol 77% EtOH and resuspended in 25 μl of TBS for SDS-PAGE analysis. The EtOH wash was pooled with the original EtOH supernatant fraction and concentrated by evaporation on a speed vac. Two low Mr glycoproteins (16 kDa and 13 kDa) were thus purified from the original glycoprotein mixture, based on their solubility in 77% EtOH.

(4) Preparation of proteins for NH$_2$-terminal amino acid sequence analysis by SDS-PAGE and electroblot transfer to Immobilon membrane

Protein samples were fractionated by Laemmli SDS-PAGE (Laemmli, 1970) and transferred to ImmobilonP or ImmobilonP-SQ membrane (Millipore), stained with coomassie blue R250 (Sigma), and individual bands cut out for protein sequence analysis (Matsudaira, 1987). Briefly, the running gel, with acrylamide (Millipore Duracryl acrylamide/bis) concentrations ranging from 6.5% - 14%, was polymerized for 48 hours and then pre-run for 1 hour at 100 V with 46 μM glutathione-reduced (Sigma) in the running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) in the upper reservoir only. A
3% stacking gel was then poured and polymerized for 1 hour. Samples were prepared for electrophoresis by boiling for 2 min in reducing sample buffer. Electrophoresis was performed at 200 V for 45-60 min with 0.1 M sodium thioglycolate (Sigma) in the upper reservoir running buffer. After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 15% MeOH) and electroblotted at 400 mA for 1 hr using a Hoeffer mini tank blot apparatus. The transfer buffer was pre-chilled to 4°C and cold water was run through the cooling chamber of the apparatus for the duration. Post-transfer, the membrane was washed for 5 min in MilliQ water (Millipore) and then stained for 2-5 min with either 0.05% or 0.1% coomassie R in 50% MeOH. The membrane was rinsed briefly with milliQ and destained with 50% MeOH for 5-10 min and allowed to air dry. Except where indicated, all of the gel reagents were from Millipore and all solutions were made with MilliQ water. Sequence analysis was performed by Sandy Kielland at the University of Victoria Microsequencing Centre.

E. Activation of rGP63s and Determination of Proteinase Activity

Purified rGP63s, 0.2 mg/ml (3.2 µM), was activated by incubation with an equal molar ratio of HgCl₂ (3.2 µM) for 10 min at 37°C and then added directly to proteinase assays. Control samples were incubated in the absence of HgCl₂. Promastigote GP63, 0.2 mg/ml, (purified from L. major LEM513 promastigotes, J. Bouvier) was also incubated for 10 min at 37°C with 3.2 µM HgCl₂. A stock of activated rGP63s was prepared by incubating 40 µg of purified rGP63s with 3.2 µM HgCl₂ for 10 min at 37°C followed by dialysis against TBS at 4°C. The depletion of HgCl₂ was verified by concentrating the dialyzed activated rGP63s by ultrafiltration on a Centricon-10 and then demonstrating the inability of the flow-through buffer to activate latent rGP63s. Proteinase activity was measured by using a solid phase gelatin substrate SDS-PAGE assay using
0.1% gelatin instead of 0.25% fibrinogen as described (Button et al., 1993) and by using both a soluble azocasein assay (Etges et al., 1986b) and a more sensitive soluble succinylated casein assay (QuantiCleave Protease Assay, Pierce).

For the azocasein assay, aliquots of rGP63s were incubated in a final volume of 100 µl with 850 µg azocasein (Sigma) in TBS at 37°C for varying time periods. Inhibition of proteinase activity was assayed by adding 1,10-phenanthroline (Sigma) to a final concentration of 5 mM diluted from a 500 mM stock in methanol. Equivalent amounts of methanol were added to assays not containing 1,10-phenanthroline. Non-hydrolyzed azocasein was removed by precipitation with an equal volume of 5% TCA for 20 min at 4°C and centrifugation for 5 min at 14,000 x g. Aliquots of the supernatant, 80 µl, were added to microtitre wells containing 20 µl of 4 N NaOH; soluble azopeptides were detected by measuring absorbance at 450 nm using a Vmax kinetic microplate reader (Molecular Devices). Non-specific hydrolysis of azocasein was determined by controls not containing rGP63s and subtracted from experimental readings. Proteinase activity was expressed as % relative activity with the maximum absorbance of activated rGP63s set at 100% as described (Springman et al., 1990; Crabbe et al., 1992). Six measurements were determined for each reaction and the means compared for statistical significance using analysis of variance according to the Keuls multiple range test.

For the succinylated casein assay (QuantiCleave Protease Assay, Pierce), aliquots of rGP63s and of promastigote GP63 were incubated for 1 hour at 37°C with succinylated casein in 50 mM borate, pH 8.5, as described in the manufacturer's protocol. Two measurements were determined for each sample and the means compared for statistical significance using analysis of variance according to the Keuls multiple range test.
F. Expression of rGP63s in COS-7 cells

(1) PCR modification of GP63 for expression in COS-7 cells

The *L. major* GP63 gene (Button and McMaster, 1988) was modified by deletion of the 3' terminus encoding the COOH-terminal GPI attachment sequence (AcPP63.3 gene) in an effort to produce a secreted protein in the baculovirus system (Button et al., 1993). This gene, referred to as wtGP63-S, did not, however, result in secretion of rGP63 from insect cells (Button et al., 1993). Further modification of the wtGP63-S gene, by replacing a portion of the *Leishmania* NH2-terminal signal peptide with a baculovirus signal peptide sequence and inclusion of a 5' Kozak sequence (AcPP63.5 gene), resulted in secretion of rGP63 in the baculovirus system (Button et al., 1993); and, this gene is referred to as GP63-S (see Figure 12, page 62). The modifications described above also introduced 5' and 3' BamHI cloning sites flanking the coding sequence of both genes. The nucleotide sequence of both genes was determined; and, as reported previously, the sequence of the wtGP63-S gene agreed with that of the cloned *L. major* gene whereas the sequence of the GP63-S gene revealed a single transition mutation of Gly-188 to Ser-188 (Button et al., 1993). This mutation is located within a non-conserved region of GP63 and presumably would not affect the structure of function of the recombinant protein as previously discussed (Button et al., 1993).

Two other GP63 genes, encoding the wildtype and the modified NH2-terminal signal sequences, were prepared to encode cell surface GPI-linked GP63 and are referred to as wtGP63-GPI and GP63-GPI, respectively. First, the *L. major* GP63 gene (Button and McMaster, 1988) was modified by PCR to introduce 5' and 3' BamHI cloning sites flanking the coding sequence as previously described using the 5'41 primer (Button et al., 1991) and the 3' 53 primer,

```
GTCCGGATCTAGAGCGCCACGGCCAGCAGCGCCAGCTGGCCACCCGGCAGCGCCCGTG
```  
(BamHI site underlined) and the resulting PCR product was cloned into Bluescript vector
To create wtGP63-GPI, a 782 bp SstI/BamHI fragment purified from the 3' end of this cloned PCR product was ligated to a 1031 bp BamHI - SstI fragment containing the 5' end of the wtGP63-S gene which encodes the wildtype NH$_2$-terminus. To create GP63-GPI, the same 782 bp SstI/BamHI 3' fragment was ligated to a 970 bp BamHI - SstI fragment containing the 5' end of the GP63-S gene which encodes the modified NH$_2$-terminus. The absence of PCR errors in the 3' 782 bp fragment was confirmed by DNA sequencing of the 3' end of the GP63-GPI gene.

Another GP63 gene, encoding the modified NH$_2$-terminus, was prepared to encode a transmembrane form of GP63, termed GP63-TM. The GP63-GPI gene was modified by replacing the COOH-terminal GPI attachment signal with a gene fragment encoding the transmembrane (20 aa) and cytoplasmic (29 aa) domain of the Vesicular Stomatitis Virus glycoprotein G (Rose and Bergmann, 1982; Berger et al., 1989). This gene fragment was isolated as a 248 bp AluI - BamHI fragment from the plasmid pSVGL (Rose and Bergmann, 1982). First, the L. major GP63 gene (Button and McMaster, 1988) was modified by PCR to remove the COOH-terminal GPI attachment sequence and to introduce an SspI restriction site (underlined) using the 3' TM primer (complementary strand) CTTCATAATTTGGCCGCGTCCCTGCGC. This primer also introduced a change of AAC (Asn-577) to CAA (Gln-577) (indicated in large capital letters) resulting in the substitution of the Asn-577 GPI addition site (Schneider et al., 1990). The L. major GP63 gene (Button and McMaster, 1988) was used as a template for PCR using the 5'41 primer and the 3' TM primer described above. A 3' 706 bp SstI - SspI fragment was purified from the PCR product and then ligated to the 5' 970 bp BamHI-SstI GP63 fragment (encoding the 5' end of the GP63-S and GP63-GPI genes) and to the 248 bp AluI-BamHI fragment encoding the VSV-G transmembrane and cytoplasmic domains. Correct ligation of the three fragments and the absence of PCR errors in the 3' 706 bp fragment was confirmed by DNA sequencing of the 3' end of the GP63-TM gene. Double stranded DNA sequencing was carried out using the dideoxy chain termination method (Sanger et al., 1977) using
Sequenase™ (US Biochemicals) as indicated by the manufacturer with the addition of 10% DMSO to the labeling reactions.

Each of the GP63 genes described above was subcloned into the transient COS cell expression vector pAX111 (Kay et al., 1990; Kay and Humphries, 1991).

(2) transfection of COS-7 cells and preparation of lysates

COS-7 cells (Gluzman, 1981) (obtained from the American Type Culture Collection, catalogue number CRL 1651 Rockville, MD.) were maintained in DMEM (Gibco BRL) + 10% FCS (HyClone). Cells were resuspended in PBS to 8 x 10^7 cells per ml and 0.8 ml transferred to a 0.4 cm cuvette (BioRAD) and transfected with 10 μg CsCl purified plasmid DNA (Sambrook et al., 1989) by electroporation at 1900 Volts and 25 μF as described (Kettelborough et al., 1991). Cells were transferred to DMEM + 10% FCS 10 min after electroporation, plated in duplicate in 100 mm tissue culture plates and incubated at 37°C. Cells were harvested 72 hr post transfection and lysates were prepared by incubating washed cells at 5 x 10^7 cells per ml in TBS containing 0.2% Zwittergent 3-14 (Calbiochem) for 10 min on ice. Lysates were then centrifuged for 10 min at 14,000 x g in a microfuge and the supernatants stored at -20°C. Lysates of L. major A2 promastigotes were prepared as above at a concentration of 1 x 10^9 cells per ml. Serum free COS-7 cell supernatants were prepared by washing cells 48 hr post transfection and then incubating in serum free DMEM for an additional 24 hr. Serum free culture supernatants were centrifuged at 2.7 x g for 10 min and then concentrated 10 fold and the buffer exchanged with 0.2% Zwittergent in TBS by ultrafiltration using a Centricon-10 (Amicon). Cell lysates and supernatants were analyzed for the presence of GP63 by Western blot analysis as described previously using monoclonal antibody #139 (Button et al., 1991). N-linked glycosylation of rGP63 was assessed by a decrease in M_r following incubation with Endoglycosidase F (EndoF) (Boehringer Mannheim). Lysates or
supernatants were incubated over night at 37°C with EndoF under reducing conditions as specified by the manufacturer.

(3) Fluorescence flow cytometry analysis

Transfected COS cells and *Leishmania* promastigotes and amastigotes were prepared for flow cytometry analysis by indirect immuno-labeling. Cells (5 x 10^6) were washed with 10%FCS/DMEM and incubated for 1 hour with 100 μl primary antibody (mAb #96 or rabbit antiserum). The cells were then washed 2 times with 10%FCS/DMEM and incubated for 40 min in 50 μl FITC-conjugated secondary antibody (FITC-goat-anti-mouse IgG or FITC-horse anti-rabbit IgG, Southern Biotech.) diluted in 10%FCS/DMEM. Cells were washed as before and resuspended in 10%FCS/DMEM for flow cytometry using a Becton Dickinson FACScan analyzer. *Leishmania* promastigotes and amastigotes were fixed in 0.4% formaldehyde after the final wash.

G. Analytical SDS-PAGE and Western Blot Analysis

Laemmli SDS-PAGE (Laemmli, 1970) and western blot analysis was performed as previously described (Wallis and McMaster, 1987). Following SDS-PAGE, proteins were transferred to Immobilon P for 40 min at 15 V using a BioRAD semi-dry transfer cell. Alkaline phosphatase-conjugated secondary antibodies were purchased from BioRAD and Southern Biotech. BCIP/NBT developing reagents were from BioRAD. Blots containing biotin-labeled proteins were developed with streptavidin alkaline phosphatase (Amersham) instead of primary and secondary antibodies.

Analytical SDS-PAGE gels were silver stained as described below. Following fixation for 30 min in 50% MeOH, 10% acetic acid, gels were rehydrated by microwaving for 1 min in 10% MeOH, 10% acetic acid, washed with dH2O for 5 min, microwaved for 1 min in 33 μM DTT and washed with dH2O. The gels were stained for 15 min with
0.1% AgNO₃, washed with dH₂O, and developed with 3% Na₂CO₃, 0.037% formaldehyde.
Chapter 3. Analysis of the Activation Mechanism and Processing of rGP63

Previous studies have shown that expression of a recombinant *L. major* GP63 gene in the baculovirus insect cell expression system resulted in the secretion of rGP63 with latent metalloproteinase activity (Button *et al.*, 1993). The proteinase activity of the baculovirus derived rGP63, referred to as rGP63s, could be activated by the mercurial compound, HgCl2 (Button *et al.*, 1993), similar to the activation of other latent metalloproteinases (Springman *et al.*, 1990; Van Wart and Birkedal-Hansen, 1990). To extend these studies the activation of rGP63 metalloproteinase activity was examined using both secreted and cell surface expression systems.

A. Results

1. activation and autocatalytic processing of rGP63s

   (a) affinity purification of rGP63s

   rGP63s expressed in the baculovirus insect cell system was purified from cell supernatant in a single step using monoclonal antibody affinity chromatography (Figure 7). A total of 1.8 mg of rGP63s was purified from 200 ml supernatant. Purified rGP63s eluate was free from contaminating proteins as detected by silver stained SDS-PAGE analysis of 0.2 μg, 0.4 μg and 1.0 μg aliquots of the eluted protein (Figure 7A, lanes 4,5,6). Western blot analysis, using an *L. major* extract as a positive control (Figure 7, lane 1), confirmed the identity of the eluted protein as GP63 (Figure 7B, lanes 4,5,6). The lower *M*<sub>r</sub> bands seen in the *L. major* extract (Figure 7B, lane 1) were likely degradation products and were observed consistently. Analysis of equal volumes of the starting supernatant (Figure 7, lane 2) and the pooled column flow through fractions (Figure 7, lane 3) demonstrated the efficient binding of GP63 to the column as GP63 was not detected in the flow through material.
rGP63s expressed in the baculovirus insect cell system was purified from cell supernatant in a single step using monoclonal antibody affinity chromatography. The starting supernatant (1 μl, lane 2), column flow through (1 μl, lane 3) and column eluate (0.2 μg, lane 4; 0.4 μg, lane 5; 1.0 μg, lane 6) were analyzed by silver stained SDS-PAGE (A) and by western blot analysis (B) using a monoclonal antibody specific for GP63. *L. major* A2 promastigote detergent extract (1.5 x 10⁶ cells per sample, lane 1) was used as a positive control for GP63 in (B). The position and apparent molecular mass (kDa) of BioRAD low range protein standards is indicated in the left margin in (A).
(b) Autolytic activation of rGP63s proteinase activity

The purified rGP63s exhibited latent proteinase activity and required activation for full enzymatic activity. Treatment of rGP63s for 10 min at 37°C with 3.2 μM HgCl₂, as described in material and methods, resulted in an enhancement of proteinase activity assayed by gelatin substrate SDS-PAGE (Figure 8B) and a concomitant 3000 M₀ decrease of rGP63s analyzed by silver stained SDS-PAGE (Figure 8A). As previously reported (Button et al., 1993), rGP63s has a lower mobility than native GP63 under the non-denaturing conditions in Figure 8B. Native GP63, treated with phosphatidylinositolphospholipase C to remove the GPI anchor, has been shown to have a similar mobility to rGP63s under non-reducing conditions (Button et al., 1993).

A soluble azocasein assay was used to quantitate the proteinase activity of rGP63s (Figure 8C). In these reactions varying amounts of rGP63s were pre-incubated for 10 min at 37°C with or without 3.2 μM HgCl₂ and then added to substrate and incubated for 24 hr at 37°C. As shown in Figure 8C, treatment with HgCl₂ resulted in a three to four fold enhancement of proteinase activity compared to treatment without HgCl₂. The proteinase activity of activated rGP63s titrated over a range of 0.02 μg to 8.0 μg and proteinase activity began leveling off at higher concentrations presumably as substrate became limiting. The level of enhancement of rGP63s proteinase activity by HgCl₂ was similar to that reported for the activation of matrix metalloproteinases (Engler et al., 1992; Crabbe et al., 1992). The concentration of HgCl₂ required for the activation of rGP63s was also titrated and ranged from 3.2 nM to 320 μM, representing 0.001 to 1000 molar equivalence, respectively, (data not shown) and activation was optimal at equimolar concentrations (3.2-μM) and higher. Inclusion of 5 mM 1,10-phenanthroline, a known inhibitor of native promastigote GP63 (Etges et al., 1986b; Chaudhuri and Chang, 1988; Etges et al., 1989), inhibited the activity of both latent and activated rGP63s (Figure 8C). The effects of HgCl₂ activation of rGP63s proteinase activity and inhibition by 1,10-phenanthroline (Figure 8C, 8 μg samples) were found to be statistically significant (p < 0.01).
Figure 8  Activation of purified latent rGP63s by HgCl\textsubscript{2} and inhibition by 1,10-phenanthroline

Purified rGP63s was incubated at 37°C for 10 min in the presence (rGP63s\textsuperscript{+}) or absence (rGP63s\textsuperscript{−}) of an equimolar ratio of HgCl\textsubscript{2} (3.2 μM). The apparent Mr of each sample was determined by silver stained SDS-PAGE analysis of 0.3 μg rGP63s\textsuperscript{−} (lane 1) and 0.3 μg rGP63s\textsuperscript{+} (lane 2) (A). The position and reported molecular mass (kDa) of protein standards (BioRAD) is indicated on the left margin in (A). In (B), the proteinase activity of 1.0 μg rGP63s\textsuperscript{−} (lane 2) and 1.0 μg rGP63s\textsuperscript{+} (lane 3) was measured by gelatin substrate SDS-PAGE. A detergent extract of L. major A2 promastigotes (lane 1, 2 x 10\textsuperscript{6} cells per sample) was used as a positive control. In (C), the proteinase activity of rGP63s\textsuperscript{−} and rGP63s\textsuperscript{+}, in the presence or absence of 5 mM 1,10-phenanthroline, was quantitated using a soluble azocasein assay as described in Materials and Methods. rGP63s samples were as follows: rGP63s\textsuperscript{−} (open circles); rGP63s\textsuperscript{−} plus 5 mM 1,10-phenanthroline (open triangles); rGP63s\textsuperscript{+}, (filled circles); rGP63s\textsuperscript{+} plus 5 mM 1,10-phenanthroline, filled triangles.
To compare the activity of activated rGP63s with purified promastigote GP63, a more sensitive soluble succinylated casein assay was used. Figure 9 shows the proteinase activity of varying amounts of rGP63s, preincubated with or without 3.2 μM HgCl₂, and of promastigote GP63 preincubated with 3.2 μM HgCl₂. The proteinase activity of promastigote GP63 was not affected by the presence of HgCl₂ (data not shown). The activity of latent rGP63s was significantly different (p < 0.01) from the activity of both activated rGP63s and promastigote GP63 (140 ng samples) whose activities were not significantly different (p > 0.01). Thus, the specific activity of HgCl₂ activated rGP63s was found to be equivalent to that of purified promastigote GP63. In this assay, the proteinase activity of rGP63s and promastigote GP63 titrated over a range of 8.75 ng to 140 ng. When assayed for 1 hr at 37 °C (Figure 9), treatment of rGP63s with HgCl₂ resulted in a two to three fold enhancement of proteinase activity compared to treatment without HgCl₂; when assayed for 20 min a five fold enhancement was seen (data not shown). The ratio of enhancement was smaller in longer assays due to spontaneous activation of the latent rGP63s over time as shown in Figure 10.

To study further the mechanism of activation, latent rGP63s was preincubated for 10 min at 37 °C, with or without 3.2 μM HgCl₂, and then 2 μg aliquots were incubated with azocasein substrate and assayed for proteinase activity over a 24 hr period (Figure 10A). Samples were also assayed by western blot analysis for conversion of the higher Mₗ latent rGP63s to the lower Mₗ activated rGP63s (Figure 10B). The proteinase activity of HgCl₂ treated rGP63s reached a plateau after 8 hr, presumably as substrate became limiting, and the level of enhancement ranged from 5 fold at 1 hr to 2 fold at 24 hr (Figure 10A). Similar to matrilysin, a matrix metalloproteinase (Crabbe et al., 1992), untreated latent rGP63s also demonstrated some proteinase activity that increased over the 24 hr incubation period (Figure 10A). The difference in activity between the latent and activated rGP63s at the 24 hr time point was found to be statistically significant (p < 0.01). Western blot analysis of aliquots of the latent rGP63s reactions, removed at each time
Figure 9  Proteinase activity of HgCl₂-activated rGP63s is comparable to native promastigote GP63

rGP63s was incubated at 37°C for 10 min with (rGP63s⁺) or without (rGP63s⁻) 3.2 μM HgCl₂ and purified promastigote GP63 was incubated with 3.2 μM HgCl₂. The proteinase activity of aliquots of each sample was determined in duplicate using a soluble succinylated casein assay as described in Materials and Methods. Proteinase aliquots of 8.75 ng, 17.5 ng, 35 ng, 70 ng, and 140 ng were assayed. rGP63s⁻ (open circles); rGP63s⁺ (filled circles); HgCl₂ treated promastigote GP63 (filled triangles). Proteinase activity is expressed as % relative activity with the maximum absorbance of rGP63s⁺ set at 100%.
Figure 10. Time course of rGP63s proteinase activity and autolytic activation of latent rGP63s

(A) Purified rGP63s was pre-incubated for 10 min 37 °C in the presence (rGP63s⁺, filled circles) or absence of 3.2 μM HgCl₂ (rGP63s⁻, open circles) and the proteinase activity of 2 μg aliquots was monitored over a 24 hr time period using a soluble azocasein assay as described in Materials and Methods. Aliquots (2.0 μg) of rGP63s⁻ mixed with a one tenth molar ratio of activated rGP63 0.2 μg, previously activated and dialyzed to remove HgCl₂, were also assayed over the 24 hr time period (X centered circles). The proteinase activity of 0.2 μg aliquots of previously activated rGP63 alone was also assayed (X). The reactions were initiated by addition of substrate and incubated at 37°C for 0, 1, 2, 4, 6, 8, and 24 hr. Proteinase activity is expressed as % relative activity with the maximum absorbance of HgCl₂ treated rGP63s set at 100%. (B) A fraction (1/50) of each proteinase reaction described in (A) was analyzed by denaturing SDS-PAGE and western blot analysis using monoclonal antibody #139 against GP63. Lanes: rGP63s⁻, 0 hr (lane 1); previously activated rGP63s, time 0 hr (lane 2); rGP63s⁺, 0 hr (lane 3); rGP63s⁻ plus 1/10 activated rGP63s, 0 hr (lane 4), 1 hr (lane 5), 2 hr (lane 6), 4 hr (lane 7), 6 hr (lane 8), 8 hr (lane 9), 24 hr (lane 10); rGP63s⁻, 24 hr (lane 11); previously activated rGP63s, 24 hr (lane 12); rGP63s⁺, 24 hr (lane 13). The position of latent rGP63s (arrow) and activated rGP63s (arrow with asterisk) is indicated on the right margin.
point, revealed the presence of increasing amounts of a lower Mr band presumably corresponding to activated rGP63s. Aliquots taken at 0 hr and 24 hr are shown in Figure 10B (lanes 1 and 11, respectively). This observation together with the activation of latent rGP63s by HgCl2 and concomitant decrease in Mr (Figures 8 & 10) suggests a mechanism of autocatalytic processing resulting in the observed decrease in Mr.

To determine whether activation of rGP63s proteinase activity may occur by a cis (intramolecular) or trans (intermolecular) autocatalytic mechanism, 2.0 µg aliquots of latent rGP63s were incubated with 0.2 µg previously activated rGP63s, dialyzed to remove HgCl2, and assayed over a 24 hr period. As shown in Figure 10A, inclusion of 0.1 molar equivalents of previously activated rGP63s to latent rGP63s did not result in an enhancement of activation of rGP63s proteinase activity. These reactions paralleled those of latent rGP63s, incubated in the absence of HgCl2, and of 0.2 µg aliquots of previously activated rGP63s incubated alone with substrate (Figure 10A); at the 24 hr time point these reactions were not significantly different (p > 0.01). As seen for untreated latent rGP63s, western blot analysis of aliquots of the mixture of activated and latent rGP63s reactions, removed at each time point (Figure 10B, lanes 4 - 10), revealed the presence of increasing amounts of a lower Mr band presumably corresponding to activated rGP63s. After 24 hr at 37 °C the proportion of higher Mr latent material remaining in the mixture of latent and activated sample (Figure 10B, lane 10) appeared identical to that in the untreated latent sample (Figure 10B lane 11). This demonstrates that addition of activated rGP63s did not promote or enhance the conversion of latent rGP63s to the active form of lower Mr (Figure 10B). These results suggest activation of rGP63s by HgCl2 may occur by an autocatalytic cis mechanism. A cis autoactivation mechanism has also been proposed for the activation of the matrix metalloproteinase human skin fibroblast procollagenase where the activation rate was also not enhanced by addition of active enzyme and was found to be independent of enzyme concentration (Stricklin et al., 1983; Grant et al., 1987).
(c) progressive removal of the rGP63s pro region upon activation with HgCl₂

To determine the processing events that resulted in the decrease in the Mₚ of the activated rGP63s following treatment with HgCl₂, latent and activated rGP63s were subjected to NH₂-terminal amino acid sequence analysis. The results (Figure 11) show that latent rGP63s contains the predicted pro region (61-63 aa). Two NH₂-terminal sequences beginning at positions 38 and 40 were found in a 3:1 ratio, respectively (Figure 11B). These two sequences correspond to the use of two different predicted signal peptidase cleavage sites (von Heinje, 1986) indicated by asterisk-arrows in Figure 11A. Three aliquots of rGP63s, incubated with HgCl₂ at 37 °C for either 10 min, 60 min, or 20 hr, were also analyzed. In each of these three activated samples, the predominant sequence obtained corresponded to the mature NH₂-terminal amino acid sequence previously determined for GP63 purified from L. major promastigotes (Button and McMaster, 1988). However, a number of intermediate sequences were also found. Following incubation with HgCl₂ for 10 min, four intermediates of various lengths were found with amino termini beginning at positions 73, 80, 84, and 86. The longest intermediate, starting at position 73, corresponds to the removal of the NH₂-terminal 33-35 amino acids of the pro region containing Cys-48 proposed to be involved in regulation of GP63 metalloproteinase activity according to the cysteine switch mechanism (Grant et al., 1992; Birkedal-Hansen et al., 1993). Following incubation with HgCl₂ for 60 min, two intermediate sequences were found, starting at positions 84 and 86. These are the same as the two shortest intermediates found after 10 min. Upon incubation for 20 hr, intermediates were found starting at positions 86 and 89. Thus, amino acid sequence analysis of rGP63s clearly shows a progressive removal of the pro region over time following activation with HgCl₂.
Figure 11 NH₂-terminal amino acid sequence of latent and HgCl₂-activated rGP63s

Purified rGP63s was incubated at 37°C in the presence of an equal molar ratio of HgCl₂ (3.2 μM) for 10 min (+HgCl₂ 10 min), 60 min (+HgCl₂ 60 min) and 20 hr (+HgCl₂ 20 hr). Immediately following incubation at 37°C, the metalloproteinase inhibitor 1,10-phenanthroline was added at a final concentration of 25 mM to the 10 min and 60 min HgCl₂ treated samples to prevent further activation during preparation of the samples for sequencing. Latent rGP63s was incubated at 37°C for 10 min in the absence of HgCl₂. The latent rGP63s sample was fractionated by SDS-PAGE, transferred to Immobilon membrane (Millipore) and the NH₂-terminal protein sequence determined as described (Beecroft et al., 1993). The three activated samples were applied to Problot membrane (Applied Biosystems) by centrifugation onto a ProSpin sample preparation cartridge at 4°C and the protein sequence determined. Only 5 cycles were determined for the 20 hr sample. The sequences obtained are shown in (B) and are numbered according to the predicted amino acid sequence of the L. major GP63 gene (Button and McMaster, 1988). The alignment of these sequences with the predicted NH₂-terminal amino acid sequence of prepro rGP63s (Button et al., 1993) is depicted by horizontal lines in (A). The NH₂-terminus of GP63 purified from L. major promastigotes is indicated by the larger sized letters starting at position 101 (Button et al., 1988). The pro region cleavage sites observed for the HgCl₂ treated samples are indicated with arrows above the sequence. The two different pre-region cleavage sites observed for the latent rGP63s sample are indicated with asterik-arrows.
A.

\[
\begin{align*}
\text{MSAILVLYVLVAVGTAAAWAHAGALQHRCVHDAMQARVRQSVADH}
\end{align*}
\]

\[
\begin{align*}
\text{KAPGA}VSAVGLPYVTLDAAHTAAADPRPGSARSVVRDV
\end{align*}
\]

B.

<table>
<thead>
<tr>
<th>NH2-Terminal Amino Acid Sequence Data †</th>
<th>rGP63s</th>
<th>rGP63s + HgCl₂ 10 min</th>
<th>rGP63s + HgCl₂ 60 min</th>
<th>rGP63s + HgCl₂ 20 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>73 AVGLPYVTLDAA 83</td>
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</tr>
<tr>
<td></td>
<td>40 HAGALQHRCVHD 51</td>
<td>80 TLDAHHTAAAAA 90</td>
<td>86 TAAAADPRP 94</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>84 AHTAAADPRP 94</td>
<td>86 TAAAADPRP 95</td>
<td>101 VRDVNWGALRI 110</td>
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† the amino acid sequences are numbered according to the predicted sequence of the *L. major* GP63 gene (Button et al, 1988)
(2) Processing of secreted and membrane rGP63 metalloproteinase expressed in COS-7 cells

Whereas rGP63s was secreted as a latent pro enzyme in the baculovirus insect cell system, native GP63 is expressed on the cell surface of *Leishmania* as an active proteinase. This difference may be due to differential autocatalytic processing resulting from conformational differences between membrane bound and secreted recombinant GP63. Alternatively, this difference may be due to differences in the processing of GP63 in *Leishmania* versus insect cells, or to differences in the intracellular pathways leading to glypiation and cell surface expression versus secretion. To address these questions, the expression and processing of membrane versus secreted forms of rGP63 (Figure 12) were compared by transient expression in COS-7 cells and the validity of the COS cell expression system was demonstrated.

(a) rGP63s is secreted from COS-7 cells as a latent proteinase

The expression of secreted rGP63s in COS-7 cells was examined and compared to rGP63s produced in the baculovirus system. As previously reported, modification of the wildtype GP63 signal peptide was found to be necessary for secretion of rGP63s in the baculovirus system (Button *et al.*, 1993). Expression of the wtGP63-S gene, containing the wildtype signal peptide (pre region) (Figure 12A), did not result in secretion of rGP63s from insect cells. However, expression of the GP63-S gene, encoding a signal peptide modified by replacing a portion of the *Leishmania* signal peptide with a baculovirus signal peptide sequence (Figure 12A) and inclusion of a 5' Kozak consensus sequence (Kozak, 1986), resulted in efficient secretion of rGP63s. Therefore, to determine if either the wildtype or modified signal peptide would be recognized in COS-7 cells, both the wtGP63-S gene and the GP63-S gene (Figure 12B) were subcloned into the COS expression vector pAX111 and expressed in COS-7 cells (see Materials and Methods). Western blot analysis of serum free culture supernatants (Figure 13) demonstrated that the expression of both
Figure 12 Schematic diagram of GP63 constructs modified for surface expression or secretion in COS-7 cells

A. The amino acid sequence of the two different amino terminal signal sequences (pre) used (Button et al., 1993). Amino acids derived from GP63 are indicated in upper case letters and amino acids derived from a baculovirus signal sequence are indicated in lower case letters. The predicted N-region (N), hydrophobic region (H) and cleavage region (C) are also indicated. The first two amino acids (HA) of the predicted pro region are also shown.

B. Schematic diagram of each GP63 gene construct used in this study. Each domain, predicted to be encoded by the constructs, is indicated with a box. The COOH-terminal signal sequence for addition of a GPI anchor is indicated as GPI. The VSV-G transmembrane and cytoplasmic domain is indicated as TM. See Materials and Methods for details of construction.
Figure 13  COS-7 cell expression of either the wtGP63-S gene or the GP63-S gene results in secretion of glycosylated rGP63s

Serum free culture supernatants of COS-7 cells transfected with pAX111 (lanes 3,4), wtGP63-S (lanes 5, 6) or GP63-S (lanes 7, 8) and cell extracts of L. major promastigotes (lanes 1, 2) were incubated overnight in the absence (-) and presence (+) of Endoglycosidase F (EndoF) and then analyzed by western blot analysis using a monoclonal antibody specific for GP63. Glycosylation of rGP63s was demonstrated by a decrease in Mr following treatment with EndoF. Culture supernatants were concentrated as described in Materials and Methods. The Leishmania cell extracts corresponded to 1.5 X 10^6 cells per sample. The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin.
wtGP63-S and GP63-S resulted in the secretion of rGP63s. The wildtype GP63 signal sequence was thus recognized in COS-7 cells; however, the modified signal sequence appeared to consistently result in a higher level of expression. As shown in Figures 13 and 14, analysis of equal volumes of culture supernatant consistently revealed higher levels of rGP63s in supernatant from cells expressing GP63-S. Size microheterogeneity was seen for rGP63s expressed from either gene (Figures 13 & 14) and may have been due to differences in glycosylation.

A decrease in M_r following treatment with Endoglycosidase F (EndoF) confirmed that the COS-7 cell rGP63s was glycosylated (Figure 13 and 21). Furthermore, similar to rGP63s secreted by the baculovirus system, the COS-7 cell recombinant protein was also secreted as a latent metalloproteinase. HgCl_2 treatment of rGP63s secreted by COS-7 cells resulted in a decrease in M_r (Figure 14B, lanes 5-8) and a concomitant activation of proteinase activity was visible for the GP63-S encoded rGP63s (Figure 14A, lanes 7,8) as determined by gelatin SDS-PAGE. The lack of detectable proteinase activity for wtGP63-S encoded rGP63s, even after HgCl_2 treatment (Figure 14A), was likely due to the lower level of rGP63s present. Also, the activation may have been incomplete as a portion of the rGP63s did not exhibit a shift in M_r (Figure 14B).

As demonstrated in Figures 8-11, the latency of the rGP63s produced in the baculovirus system has been correlated with retention of the pro region. Although isolation of sufficient quantities of rGP63s for NH_2-terminal sequence analysis was not feasible in the COS cell expression system used, the presence of at least a portion of the pro region was confirmed by western blot analysis using a polyclonal rabbit antiserum specific for the GP63 prepro peptide (Figure 15). As shown by western blot analysis in Figure 15A, this rabbit serum reacts with E. coli rGP63 encoding preproGP63 (lane 2) and proGP63 (lane 3) but not with E. coli rGP63 encoding only the mature protein (lane 4) nor with GP63 in lysates of L. major promastigotes (lane 1). However, this anti-prepro serum reacts with rGP63s in culture supernatants of COS-7 cells transfected with GP63-S
Figure 14  

**HgCl\textsubscript{2} activation of rGP63s produced in COS-7 cells**

The effects of HgCl\textsubscript{2} treatment on rGP63s in serum free culture supernatants of transfected COS-7 cells, and on purified *L.*major promastigote GP63, were examined with respect to apparent molecular mass (B) and proteinase activity (A). Samples were incubated for 10 min at 37\textdegree C in the presence (+) or absence (-) of 5 \textmu M HgCl\textsubscript{2} and then analyzed both by western blot analysis, using a mAb specific for GP63 (B), and by gelatin substrate SDS-PAGE (A). COS-7 cells were transfected with either the pAX111 vector (lanes 3, 4), the wtGP63-S gene (lanes 5, 6), or the GP63-S gene (lanes 7, 8). Purified *L.*major promastigote GP63 (lanes 1, 2) was provided by Jacques Bouvier. The position and reported molecular mass (kDa) of pre-stained protein standards is indicated in the left margin in (B).
Figure 15 Western blot analysis using an anti-prepro rabbit serum and a mAb specific for promastigote GP63

(A) The specificity of the anti-prepro serum for the GP63 prepro region was examined by western blot analysis of L. major lysate (lane 1); lysates of E. coli expressing either recombinant preproGP63 (lane 2), proGP63 (lane 3), or mature GP63 (lane 4). (B) The reactivity of the anti-prepro serum to rGP63s was examined by western blot analysis: L. major lysate (lane 1); lysate of E. coli expressing recombinant proGP63 (lane 2), serum-free culture supernatant of COS-7 cells transfected with GP63-S (lane 3). Duplicate western blot analysis using an anti-GP63 mAb confirmed the presence of GP63 in each sample in both (A) and (B). The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin in (A) and (B).
(Figure 15B, lane 3) and also with rGP63s produced in the baculovirus system (data not shown). Furthermore, the rGP63s produced by expression of the GP63-S gene in COS cells has a higher \( M_r \) than promastigote GP63, even after treatment with EndoF (Figure 13 and 21). This is also consistent with retention of the pro region. It should be noted, however, that a band of rGP63s with \( M_r \) similar to promastigote GP63 is visible following EndoF treatment of supernatant from COS cells expressing the wtGP63-S gene in Figure 13, lane 6. This could be attributed to the autoactivation of a portion of the rGP63s present. Together, the results in Figures 13, 14 and 15 suggest that rGP63s secreted by COS-7 cells was synthesized containing the pro region and was processed following treatment with HgCl\(_2\). Secretion of rGP63s as a latent metalloproteinase is thus not unique to the insect cell expression system.

(b) wildtype GP63 (rGP63gpi) is expressed as an active proteinase on the surface of COS-7 cells

The expression and processing of cell surface rGP63 was also examined in COS-7 cells. As described in Materials and Methods, the original \( L. \) major GP63 clone was modified by PCR for subcloning into the pAX111 expression vector and this gene is termed wtGP63-GPI (Figure 12B). A derivative of this gene, termed GP63-GPI (Figure 12B), was also constructed to contain the modified signal sequence encoded by the GP63-S gene. Analysis by flow cytometry demonstrated that both wtGP63-GPI and GP63-GPI directed cell surface expression of recombinant GP63 (rGP63gpi) in COS-7 cells (Figure 16). However, expression of the GP63-GPI gene consistently resulted in higher levels of expression. Approximately 44% of the cells transfected with GP63-GPI were labeled with monoclonal #96 antibody, specific for \( L. \) major GP63, whereas only 13% of the cells transfected with the wtGP63-GPI were labeled (Figure 16). Similar to rGP63s, the rGP63gpi expressed by both wtGP63-GPI and GP63-GPI transfected cells, was glycosylated, as indicated by a shift in \( M_r \) following treatment with EndoF (Figure 17).
Figure 16  Cell surface expression of rGP63gpi on transfected COS-7 cells

COS-7 cells transfected with either pAX111 vector, wtGP63-GPI or GP63-GPI were analyzed for cell surface expression of rGP63 by flow cytometry using monoclonal antibody #96 specific for GP63 and a FITC-conjugated secondary antibody (see Materials and Methods). The relative cell number is indicated on the y axis and the fluorescence is indicated on the x axis (log scale).
Figure 17  rGP63gpi produced in COS-7 cells is glycosylated

Glycosylation of rGP63gpi was demonstrated by a decrease in Mr following treatment with EndoF. Equivalent amounts of lysates of COS-7 cells transfected with pAX111 (lanes 3, 4), wtGP63-GPI (lanes 5, 6), or GP63-GPI (lanes 7,8) and cell extracts of L. major promastigotes (lanes 1, 2) were incubated overnight in the absence (-) and presence (+) of EndoF and then analyzed by western blot analysis using a monoclonal antibody specific for GP63. The Leishmania cell extracts corresponded to 1.5 X 10^6 cells per sample and the COS-7 cell extracts corresponded to 10^5 cells per sample. The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin.
A band with the same Mr as deglycosylated promastigote GP63 was seen in Figure 17 in lysates of COS-7 cells transfected with the pAX111 vector (lanes 3,4) thereby complicating interpretation of the results. However, a decrease in the proportion of the higher Mr rGP63gpi present in the untreated lysates in Figure 17 (lanes 5, 7) is clearly seen following treatment with EndoF. In other experiments, no bands were detected in the vector controls (e.g. see Figures 21, 22).

In contrast to rGP63s, the rGP63gpi encoded by wtGP63-GPI and GP63-GPI appeared to be produced as an active proteinase. Western blot and gelatin SDS-PAGE analysis of lysates from COS-7 cells expressing either wtGP63-GPI (Figure 18, lane 4) or GP63-GPI (Figure 18, lane 5) showed significant levels of rGP63gpi proteinase activity. Purified promastigote GP63 (Figure 18, lane 1) and lysates of L. major (Figure 18, lane 2) were used as positive controls. Lysates of COS-7 cells transfected with the pAX111 vector were used as a negative control (Figure 18, lane 3). Furthermore, the proteinase activity of rGP63gpi from expression of either the wtGP63-GPI gene or the GP63-GPI gene was not affected by treatment of HgCl2 (data not shown). Expression of GP63 as an active proteinase therefore does not appear to require a factor(s) unique to Leishmania.

(c) A transmembrane fusion protein of GP63 (rGP63tm) is expressed as an active proteinase on the surface of COS-7 cells

To determine if production as an active protease is linked specifically to the GPI pathway, a transmembrane form of GP63, termed GP63-TM (Figure 12B), was also constructed and expressed in COS-7 cells. Since recognition of the modified signal sequence had been demonstrated for COS cell expression of both GP63-S and GP63-GPI, and for consistency with the baculovirus rGP63s, GP63-TM was designed to encode the same modified NH2-terminal signal sequence. As described in Materials and Methods, GP63-TM was constructed by replacing the COOH-terminal signal sequence for glypiation with the transmembrane and cytoplasmic domain of the VSV-G protein. This strategy was
Figure 18  rGP63gpi is produced as an active proteinase in COS-7 cells

The proteinase activity of rGP63gpi in lysates of transfected COS-7 cells was demonstrated by gelatin substrate SDS-PAGE (A) and duplicate non-denaturing western blot analysis (B). Purified promastigote GP63 (lane 1), L. major promastigote lysate (lane 2), cell lysates of COS-7 cells transfected with either pAX111 vector (lane 3), wtGP63-GPI (lane 4), or GP63-GPI (lane 5). COS-7 cells per sample: 5 X 10^4.
previously reported to convert expression of placental alkaline phosphatase as a GPI-linked protein to a transmembrane protein in COS cells (Berger et al., 1989). Western blot analysis, using a monoclonal antibody specific for the VSV-G transmembrane region, demonstrated expression of the VSV-G transmembrane peptide in rGP63tm in lysates of transfected COS-7 cells (Figure 19, lane 2). Lysates of COS cells transfected with either the VSV-G gene (Figure 19, lane 1) or the GP63-GPI gene (Figure 19, lane 3) were used as positive and negative controls, respectively. Expression of GP63-TM and GP63-GPI resulted in similar levels of cell surface expression as determined by flow cytometry; 38% and 44%, respectively, of transfected cells were labeled with monoclonal #96 in Figure 20. Similar to rGP63 produced by GP63-GPI (Figure 21, lanes 4 & 5) and GP63-S (Figure 21, lanes 8 - 11), the rGP63 produced by expression of GP63-TM (Figure 21 lanes 6 & 7) was also glycosylated as indicated by a decrease in Mr following treatment with EndoF. As expected, rGP63tm was found to have a higher Mr than rGP63gpi. The apparent higher Mr of rGP63gpi (lane 4), as compared to promastigote GP63 (lane 1), was likely due to differences in glycosylation. Following treatment with EndoF, the Mr of rGP63gpi appears similar to promastigote GP63.

Lysates from COS-7 cells expressing either GP63-TM (Figure 22, lanes 6 &7) or GP63-GPI (Figure 22, lanes 4 & 5) showed high levels of proteinase activity without the requirement for activation by HgCl2. Treatment of either sample with HgCl2 (Figure 22A, lanes 5 and 7) did not result in a decrease in Mr or an enhancement of proteinase activity characteristic of rGP63s (Figure 22). Titration of the GP63-GPI and GP63-TM lysates to a barely detectable level of activity on gelatin SDS-PAGE still did not reveal any enhancement of activity upon treatment with HgCl2 (data not shown), thereby demonstrating that the results in Figure 22B were not an artifact of overloading the assay system. The results in Figure 22 suggest that the pro region of GP63-GPI and GP63-TM was removed during synthesis in COS-7 cells and that these membrane forms of GP63 did not require activation for full proteinase activity. This is similar to the GP63 activity seen
Expression of the VSV-G transmembrane peptide in rGP63tm was confirmed in lysates of COS-7 cells transfected with GP63-TM (lane 2). Lysates of Cos-7 cells transfected with either pSVGL (lane 1) or GP63-GPI (lane 3) served as a positive and negative controls, respectively. The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin. Cos-7 cell lysates corresponded to $10^5$ cells per sample.
Figure 20  rGP63tm and rGP63gpi are expressed at similar levels on transfected COS-7 cells

COS-7 cells transfected with either pAX111 vector, GP63-GPI or GP63-TM were analyzed for cell surface expression of rGP63 by flow cytometry using monoclonal antibody #96 specific for GP63 and a FITC-conjugated secondary antibody (see Materials and Methods). The relative cell number is indicated on the y axis and the fluorescence is indicated on the x axis (log scale).
Figure 21  EndoF analysis of rGP63tm produced in COS-7

Glycosylation of rGP63 was demonstrated by an increase in Mr following EndoF treatment of lysates of transfected COS-7. Samples were incubated overnight in the absence (-) or presence (+) of EndoF and then analyzed by western blot analysis using a mAb specific for GP63. Cell lysates of *L. major* promastigotes (lanes 1, 2); cell lysates of COS-7 cells transfected with either pAX111 vector (lane 3), GP63-GPI (lanes 4, 5), GP63-TM (lanes 6, 7), or GP63-S (lanes 8, 9); culture supernatants of COS-7 cells transfected with GP63-S (lanes 10, 11). Cells per sample: 3.3 X 10^5 (lanes 1, 2), 1.7 X 10^4 (lanes 3-7). Concentrated (10 fold) supernatant per sample: 3 μl (lanes 10, 11). The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin.
Figure 22  Effect of HgCl₂ treatment on membrane versus secreted rGP63 produced in COS-7 cells

Membrane and secreted rGP63 produced in COS-7 cells, and *L. major* promastigote GP63, were treated with HgCl₂ and then analyzed with respect to apparent molecular mass (A) and proteinase activity (B). Samples were incubated for 10 min at 37°C in the presence (+) or absence (-) of 20 μM HgCl₂ and then subjected to denaturing SDS-PAGE and western blot analysis, using mAb #139, (A) and gelatin substrate SDS-PAGE (B). Cell lysates of *L. major* promastigotes (lanes 1, 2); cell lysates of COS-7 cells transfected with either pAX111 vector (lane 3), GP63-GPI (lanes 4, 5), GP63-TM (lanes 6, 7), or GP63-S (lanes 8, 9); culture supernatants of COS-7 cells transfected with GP63-S (lanes 10, 11). Cells per sample: 3.3 X 10⁵ (A, lanes 1, 2), 1.7 X 10⁴ (A, lanes 3-7), 5 X 10⁵ (B, lanes 1, 2), 5 X 10⁴ (B, lanes 3-7). Concentrated (10 fold) supernatant per sample: 3 μl (A, lanes 10, 11), 15 μl (B, lanes 10,11). The position and reported molecular mass (kDa) of pre-stained protein standards is indicated in the left margin in (A).
in *L. major* promastigote lysates; treatment with HgCl₂ did not result in enhancement of GP63 proteinase activity (Figure 22B lanes, 1 & 2) or a decrease in Mr (Figure 22A, lanes 1 & 2). Moreover, these results suggest that expression of rGP63 as an active protease is not linked specifically to the GPI-pathway; rGP63 is also produced as an active protease when expressed as a transmembrane fusion protein.

**B. Discussion**

The similarity of the active site of GP63 and matrix metalloproteinases to bacterial thermolysin is a common feature among zinc metalloproteinases (Vallee and Auld, 1992). Mammalian matrix metalloproteinases have also been reported to be synthesized as latent enzymes requiring activation for proteinase function via the cysteine switch mechanism (Springman *et al.*, 1990; Van Wart and Birkedal-Hansen, 1990; Grant *et al.*, 1992; Birkedal-Hansen *et al.*, 1993). The results in Figures 8-11 clearly demonstrate that rGP63s, synthesized by the baculovirus system, is secreted as a zymogen requiring activation for full proteinase activity and autolytic removal of the predicted propeptide. Similarly, the results in Figures 8, 9 and 10 suggest that rGP63s secreted by COS-7 cells was also synthesized as a latent proenzyme, processed following activation with HgCl₂. Secretion of rGP63s as a latent metalloproteinase is thus not unique to the insect cell expression system.

The activation mechanism of latent rGP63s has been proposed to follow the cysteine switch pathway (Bouvier *et al.*, 1990; Button *et al.*, 1993). According to this model, latency is maintained by blockage of the active site by the interaction between the sulphhydryl group of a Cys residue in the pro region and the zinc ion in the catalytic site (see Figure 6). Conditions which promote dissociation of the Cys from the active site zinc ion lead to activation and subsequent autolytic cleavage of the propeptide. Mercurial compounds have been reported as potent activators of latent matrix metalloproteinases
GP63 contains a conserved Cys residue (Cys-48) in the pro domain 53 amino acids prior to the mature NH₂-terminus that likely interacts with the active site zinc in the latent precursor form of GP63. As shown in Figure 11, this conserved Cys residue is contained within the portion of the pro region initially removed upon incubation with HgCl₂ for 10 min and the activated rGP63s was shown to have the same specific activity as native promastigote GP63 (Figure 9). The cleavage sites observed for the GP63 pro region in Figure 11 are consistent with the peptide substrate specificity of purified promastigote GP63. Bouvier et al. (1990) found the specificity to be determined largely by the P1' site of tested peptide substrates with a preference for hydrophobic amino acids although polar residues at the P1' site were also observed. Furthermore promastigote GP63 was shown to cleave a synthetic peptide spanning the mature cleavage site of the pro peptide (ARSV ↓ VRDVN). Thus, the activation mechanism of rGP63s is consistent with the cysteine switch pathway described for matrix metalloproteinases. The cysteine switch activation mechanism therefore appears to be found from protozoa to mammals.

The concomitant removal of the GP63 pro region and activation of proteinase activity (Figures 3,4,5,6) directly demonstrates a role for the predicted pro region in regulating GP63 proteinase activity. The progressive removal of the pro region over time as shown in Figure 11 is similar to that observed for mammalian matrix metalloproteinases such as human skin fibroblast procollagenase (Grant et al., 1987), stromelysin (Nagase et al, 1990) and human matrilysin (Crabbe et al., 1992) following activation with mercurial compounds. In each case, an intermediate is generated relatively rapidly by an initial intramolecular (cis) cleavage of the proximal end of the pro region followed by a more gradual conversion to the mature form of the enzyme upon extended incubation. Cleavage of the intermediates may then occur by either cis or trans (intermolecular) interactions (Nagase et al., 1990; Suzuki et al., 1990; Crabbe et al., 1992; reviewed by Birkedal-Hansen et al., 1993). The spontaneous activation of latent rGP63s seen upon extended
incubation at 37°C (Figure 10), in the absence of HgCl₂, is also similar to that reported for matrilysin (Crabbe et al., 1992). The results in Figure 10 suggest further that the autolytic processing of the GP63 pro region is initiated by a cis mechanism since the addition of active rGP63s did not lead to an enhancement of latent rGP63s proteinase activation.

Similarly, the activation rate of proMMPs such as human skin fibroblast procollagenase (FIB-CL) (Grant et al., 1987; Stricklin et al., 1983) and proMMP-9 (Okada et al., 1992) are not enhanced by addition of active enzyme. However, the possibility that the presence of excess substrate (i.e. azocasein) competitively inhibited trans activation of latent GP63 must also be considered. Incubation of latent GP63 with activated GP63 in the absence of exogenous substrate would resolve this question.

Sequence comparison of sixteen mammalian matrix metalloproteinases has revealed a highly conserved consensus sequence, PRCGVPDV, surrounding the pro region Cys and located 2 - 4 amino acids upstream of the mature NH₂-terminus (Van Wart and Birkedal-Hansen, 1990; Park et al., 1991; Birkedal-Hansen et al., 1993). While the Cys residue within this "autoinhibitor" sequence is predicted to maintain latency by coordinating the active site zinc as described above, site directed mutagenesis of the MMP transin (rat stromelysin) has demonstrated a role for the surrounding residues (Park et al., 1991; Sanchez-Lopez et al., 1988). Whereas wildtype transin is secreted as a latent proenzyme, a number of different single amino acid substitutions result in secretion as an active enzyme. Some residues within the autoinhibitor region were shown to be sensitive to even conservative amino acid substitutions such as mutation of Val to Ile. However, the Arg residue was the only amino acid, in addition to Cys, which appeared to be essential for maintaining latency. A synthetic peptide PRCGVPDV was also shown to inhibit transin better than cysteine alone further indicating a role for the surrounding amino acids. Moreover, substitution of the Cys residue with a Ser residue abolished the inhibitory effect of the peptide.
Based on an analysis of transin substrate specificity and the effects of the various mutants, the PRCGVPDV sequence does not appear to mimic known substrates. Park et al. (1991) favour a model in which the conserved Arg residue interacts with the catalytic Glu in the active site, the Cys coordinates the active site Zn$^{2+}$, and the residues distal to Cys interact with an "inhibitor pocket" that overlaps with but is distinct from the substrate binding pocket. In this model, the inhibitor pocket consists of residues highly conserved among the MMPs but not involved in substrate specificity. Thus, MMPs with different substrate specificities could all be inhibited by the same peptide. The extensive sequence similarity shared among the mammalian MMPs (Birkedal-Hansen et al., 1993) is consistent with this model. Furthermore, as predicted by this model, the PRCGVPDV synthetic peptide has also been shown to inhibit sea urchin enamelysin, a recently identified MMP (Nomura and Suzuki, 1993). Similarly, a synthetic peptide spanning this sequence has been shown to inhibit human type IV collagenase (MMP-2) (Stetler-Stevenson et al., 1991).

The corresponding sequence within the L. major GP63 pro region, HRCVHDAM, which is located approximately 47 amino acids upstream of the mature NH$_2$-terminus, does not conform to the mammalian autoinhibitor consensus sequence. However, it does contain the putative essential ArgCys pair. Moreover, the substitutions tested for transin (Park et al., 1991; Sanchez-Lopez et al., 1988) did not include those seen for the L. major GP63 sequence. The model proposed by Park et al. (1991) could therefore be applied to this sequence. However, given that the sequence similarity between GP63 and mammalian MMPs is limited to the active site consensus sequence, HExxH, a model predicting a distinct specificity for the GP63 inhibitor pocket would be favoured. If so, a synthetic peptide HRCVHDAM might inhibit GP63 but not transin or other mammalian MMPs. Conversely, PRCGVPDV which does inhibit transin, might not inhibit GP63. As shown in Table 2, the Leishmania HRCVHDAM sequence is conserved with few variances among seven GP63 genes sequenced to date. Notably, the Arg residue is substituted with a His
Table 2  Alignment of GP63 sequences surrounding the proposed regulatory cysteine

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**MMP consensus sequence**

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residue in *L. guyanensis* GP63. Although mutation of Arg to Lys in the transin PRCGVPDV sequence resulted in loss of latency, mutation to His was not examined.

The activation intermediates observed for HgCl2 treated rGP63s all lack the HRCVHDAM sequence. In contrast, the activation intermediates observed for FIB-collagenase (Grant *et al.*, 1987), type IV collagenase (MMP-9) (Tschesche *et al.*, 1992) and matrilysin (Crabbe *et al.*, 1992) retain the PRCGVPDV autoinhibitor sequence and yet are not able to maintain latency. Moreover, the mature form of MMP-9 was found to retain the autoinhibitor sequence at its NH2-terminus (Okada *et al.*, 1992; Tschesche *et al.*, 1992). Together these data suggest that for these MMPs the NH2-terminal portion of the pro region is necessary for latency and may serve to maintain a conformation which promotes interaction of the autoinhibitor sequence with the active site. This is in contrast to observations that free synthetic peptides mimicking the autoinhibitor sequence are effective inhibitors of some MMPs (Park *et al.*, 1991; Nomura and Suzuki, 1993; Stetler-Stevenson *et al.*, 1993).

The analysis of the activation mechanism of rGP63s in Figures 3-6 appears to be the same as reported for matrix metalloproteinases (Van Wart and Birkedal-Hansen, 1990; Grant *et al.*, 1992). A number of criteria, as listed in Table 3, have been suggested for assignment of metalloproteinases to the MMP family (Nagase *et al.*, 1992). The first five criteria are intended to provide simple biochemical tests for preliminary assignment of an enzyme to the MMP family. Criteria 6 - 12 describe properties that are displayed by several members of the MMP family but have not been established for all MMPs. Assignment to the MMP family therefore does not require that these additional criteria (6-12) all be met although a narrower definition of the term MMP excludes proteinases that do not share homology to collagenase. As summarized in Table 3, rGP63s has been shown to meet 4 of the first 5 criteria (criteria 1,2,3,5). Together with the analysis of the GP63 active site (McMaster *et al.*, 1994; Macdonald *et al.*, submitted) and with previous studies of promastigote GP63 establishing criteria 7, 9 &10, this study provides strong support for
Table 3  Similarity of GP63 to the matrix metalloproteinase family

<table>
<thead>
<tr>
<th>criteria for assignment of a proteinase to the MMP family*</th>
<th>native GP63</th>
<th>rGP63s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. proteinase activity is blocked by 1,10-phenanthroline</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>2. appears in a latent form</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>3. the latent form can be activated by mercurial compounds</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>4. activity is inhibited by the tissue inhibitor of metalloproteinases (TIMP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. hydrolyzes at least one component of the extracellular matrix</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>6. calcium ion is required for activity/stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. zinc is as intrinsic metal ion</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>8. gene structure shows homology to the collagenase gene</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>9. α2-macroglobulin is inhibitory</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>10. acts extracellularly or at the cell surface</td>
<td>√</td>
<td>n.a.</td>
</tr>
<tr>
<td>11. low concentrations are present in tissues</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>12. activity is difficult to extract from tissues</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

* The criteria are as suggested by Nagase et al., 1992. Criteria that have been met are indicated by √, criteria that have not been met are indicated by X, criteria that have not been determined are left blank, and criteria that are not applicable to GP63 are indicated as n.a. References for data pertaining to native GP63 are as follows:
  - criterion 1: Etges et al., 1986b and 1989; Chaudhuri and Chang, 1888
  - criterion 2: Bouvier et al., 1989
  - criteria 9 & 10: Heumann et al., 1989
assignment of GP63 to the matrix metalloproteinase family according the broad definition of Nagase et al. (1992).

Sequence comparison of GP63 with the extended active site consensus sequences for the mammalian MMP family and the astacin family (Table 4) demonstrates that the GP63 sequence does not fit either family exactly but does share one additional residue in common with the astacin family (F-272 in GP63). Notably, GP63 does not contain the conserved third His residue found in the metzincins (Bode et al., 1993). Apart from the active site residues HExxHxxG (Table 4), there is no sequence similarity between GP63 and the MMPs. Thus, GP63 and the family of mammalian MMPs may be an example of convergent evolution analogous to the example of chymotrypsin and subtilisin (Hartley, 1979). Alternatively, GP63 may be part of the divergent zincins superfamily, perhaps representing a third sub-family distinct from both the metzincins and the gluzincins (Bode et al., 1993). Elucidation of the relationship of GP63 to the zincins will require determination of the GP63 structure by X-ray crystallography or NMR.

Unlike the mammalian MMPs which are secreted as latent enzymes, *Leishmania* GP63 is expressed on the cell surface of promastigotes as an active proteinase. A mammalian MMP that is an integral membrane protein has recently been identified and has been termed MT-MMP for membrane type MMP (Sato et al., 1994). Thus native GP63 might be considered a second example of an MT-MMP. Other known examples of cell surface Zn metalloproteinases such as neutral endopeptidase (Letarte et al., 1988) and members of the astacin family of proteinases (Dumermuth et al., 1991) do not appear to belong to the MT-MMP family. Neutral endopeptidase and the astacins do not contain the conserved MMP autoinhibitor pro region sequence and do not appear to be activated by the cysteine switch mechanism. Furthermore, neutral endopeptidase does not degrade matrix components.

In addition to the role of mammalian MMPs in ECM degradation, recent reports indicate that MMPs may also activate release of TNF-α which is produced as a membrane
Table 4  Alignment of the GP63 active site with the MMP and astacin family consensus sequences

<table>
<thead>
<tr>
<th>MMP consensus sequence</th>
<th>H E x G H x x G x x H x</th>
</tr>
</thead>
<tbody>
<tr>
<td>astacin consensus sequence</td>
<td>H E x x H x x G F x H E</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence Information</th>
<th>Consensus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. major (gene 1)</td>
<td>264 H E m a H a l G F s g p</td>
<td>275</td>
<td>1. Park et al., 1991</td>
</tr>
<tr>
<td>L. major (gene 6)</td>
<td>264 H E m a H a l G F s g p</td>
<td>275</td>
<td>2. Dumermuth et al., 1991</td>
</tr>
<tr>
<td>L. donovani</td>
<td>264 H E m a H a l G F s v v</td>
<td>275</td>
<td>3. Button and McMaster, 1988</td>
</tr>
<tr>
<td>L. chagasi</td>
<td>264 H E m a H a l G F s v g</td>
<td>275</td>
<td>4. Voth and McMaster, unpublished</td>
</tr>
<tr>
<td>L. chagasi (log)</td>
<td>264 H E m a H a l G F s v g</td>
<td>275</td>
<td>5. Webb et al., 1991</td>
</tr>
<tr>
<td>L. chagasi (stat)</td>
<td>264 H E m a H a l G F s v g</td>
<td>275</td>
<td>6. Miller et al., 1990</td>
</tr>
<tr>
<td>L. chagasi (const)</td>
<td>264 H E m a H – v G F s g t</td>
<td>275</td>
<td>7. Ramamoorthy et al., 1992</td>
</tr>
<tr>
<td>L. mexicana (C-1)</td>
<td>264 H E m a H a v G F s g t</td>
<td>275</td>
<td>8. Medina-Acosta et al., 1993</td>
</tr>
<tr>
<td>L. guyanensis</td>
<td>264 H E v a H a l G F s s t</td>
<td>275</td>
<td>9. Steinkraus and Langer, 1992</td>
</tr>
</tbody>
</table>
bound precursor that is released in its mature form by proteolytic cleavage (Gearing et al., 1994; McGeehan et al., 1994). TNF-α is a potent inflammatory mediator and has been shown to activate killing of L. major amastigotes by murine peritoneal macrophages (Liew et al., 1990c; Stenger et al., 1991; Deng et al., 1993). Thus, it would be interesting to determine if GP63 is able to activate TNF-α release as demonstrated for mammalian MMPs.

In contrast to rGP63s secreted in either the baculovirus or COS cell system, Leishmania GP63 is expressed on the cell surface of promastigotes as an active proteinase. Similarly, rGP63 expressed on the surface of COS-7 cells as either a GPI linked or a transmembrane metalloproteinase appeared to be expressed as an active proteinase and approximated native GP63 (Figures 16-22). Expression as an active proteinase, therefore, is not linked specifically to the GPI pathway nor does it require a factor or microenvironment unique to Leishmania. Perhaps activation is mediated by a processing enzyme or microenviroment localized to the cellular pathway for membrane expression versus secretion. However, it is not clear whether or not these pathways are indeed divergent. Alternatively, conformational differences between membrane and secreted rGP63 may account for the observed differences in processing in COS-7 cells. Perhaps insertion in a lipid membrane provides the necessary conformation to initiate autocatalytic removal of the pro region. Lipid association has been reported to be a requirement for the activity of tissue factor, a transmembrane glycoprotein which forms a complex with the plasma protein factor VII(a) and is involved in the initiation of blood coagulation (Paborsky et al., 1991). Substitution of the transmembrane domain with a GPI anchor did not appear to affect the activity of recombinant tissue factor whereas deletion of the transmembrane domain resulted in an inactive secreted form.

It has not been shown if rGP63gpi and rGP63tm produced in COS cells have the same NH₂-terminus as native GP63 purified from Leishmania. Expression of GP63 as an active surface proteinase in COS cells and in Leishmania might be achieved by different
mechanisms. The ability of HgCl₂-activated rGP63s to cleave its own pro region has been demonstrated (Figure 11), however there is also the possibility that *Leishmania* may contain a specific enzyme responsible for the activation of GP63 in vivo. Perhaps the proximal portion of the pro region is cleaved producing an active intermediate, thereby initiating a subsequent autolytic processing event(s) generating the mature NH₂-terminus.

A similar multi-step activation mechanism has been proposed for a number of mammalian matrix metalloproteinases and can be demonstrated *in vitro* (reviewed by Birkedal-Hansen et al., 1993). For example, proteinases such as plasmin and plasma kallikrein have been shown to mediate an initial activation of stromelysin by cleavage of a portion of the propeptide which is then followed by autolytic processing to generate the mature NH₂-terminus (Nagase et al., 1990).

Transfection of the *L. major* GP63-ASP265 active site mutant (McMaster et al., 1994; Macdonald et al., submitted) into *Leishmania* may provide further information on the activation mechanism of GP63 metalloproteinase in *Leishmania*. rGP63 encoded by GP63-ASP265 lacks proteinase activity and therefore cannot be processed autolytically. The processing and activation of GP63-ASP265-encoded rGP63 could thus be followed in the presence and absence of specific proteinase inhibitors to determine the presence or absence of heterologous processing enzymes in *Leishmania*. Transfection of the GP63-ASP265 into a mutant *Leishmania* strain in which the GP63 locus has been deleted or disrupted, would rule out the possibility of trans-activation by endogenous GP63. Moreover, a homozygous GP63-ASP265 strain would enable direct examination of the important of GP63 proteinase activity in the parasite life cycle both *in vitro* and *in vivo*.
Chapter 4. Identification of Novel Amastigote Glycoproteins

There is considerable evidence that *Leishmania* cell surface molecules play critical roles in the initiation and establishment of macrophage infection, evasion of non-specific immune defenses, and in stimulation of a cellular and humoral immune response. Relative to the promastigote, the amastigote cell surface remains relatively uncharacterized. GP63, the major surface glycoprotein on promastigotes, was studied extensively in Chapter 3. Analysis of the amastigote cell surface may lead to a better understanding of persistence of the amastigote within the host macrophage and may also lead to identification of novel antigens important to the host immune response to *Leishmania*. Amastigote surface molecules could also be important targets for both chemotherapeutic strategies and vaccine development. The potential of glycoprotein purification to enrich for cell surface proteins was therefore exploited in a search to identify novel amastigote surface molecules. The specific objectives of this study were as follows:

(i) to determine the suitability of *in vitro* amastigotes for characterization of proteins expressed *in vivo*;

(ii) to demonstrate the efficacy of lectin affinity chromatography to enrich for cell surface molecules;

(iii) to identify unique amastigote glycoproteins by NH2-terminal amino acid sequence analysis.

*L. mexicana* was chosen because it provides the advantage of an *in vitro* grown amastigote model thus making feasible the purification of microgram quantities of protein for study.
A. Results

(1) suitability of an *L. mexicana in vitro* amastigote model for characterization of amastigote proteins

The suitability of *L. mexicana in vitro* amastigotes, as a source of proteins expressed *in vivo*, was examined. Cell surface antigenic differences between *in vitro* amastigotes and promastigotes were investigated by flow cytometry using a rabbit antiserum raised to *in vivo* grown lesion *L. mexicana* amastigotes. The anti-amastigote serum was shown to specifically label the surface of *in vitro* grown amastigotes; 99% of amastigotes compared to 41% of promastigotes were labeled with a relative fluorescence greater than 110 units (Figure 23). In negative controls using NRS, 10% of amastigotes and 5% of promastigotes were labeled with this intensity. As shown in Figure 23, serum absorbed with 5x10^8 promastigotes per ml labeled 34% of amastigotes compared to 7% of promastigotes with a relative fluorescence greater than 110 units. Thus, reactivity of the serum to promastigotes was removed by absorption leaving an amastigote-specific serum. Furthermore, Figure 23 demonstrates that the *in vitro* grown amastigotes share antigenic determinants with their *in vivo* grown counterparts.

Differences between GP63 in lysates of *in vitro* amastigotes and promastigotes were demonstrated by western blot analysis using 2 different mAbs raised to *L. major* GP63 (Figure 24). Wheras mAb 235 reacted with GP63 in both promastigotes and amastigotes (Figure 24A, lanes 1 and 2, respectively), mAb 139 reacted only with promastigote GP63 (Figure 24B, lane 1). In addition, the amastigote GP63 appeared to have a higher M_r. These results are consistent with reports that *L. mexicana in vivo* amastigotes express a different form of GP63 than promastigotes (Medina-Acosta *et al.*, 1989; Frommel *et al.*, 1990; Ilg *et al.*, 1993). Together, Figures 23 and 24 demonstrate that *in vitro* amastigotes provide a feasible approach to purification of amastigote proteins expressed *in vivo*. 
Figure 23 Cell surface labeling of promastigotes and in vitro amastigotes with an anti-amastigote rabbit serum

Cell surface antigenic differences between *L. mexicana* promastigotes and *in vitro* amastigotes were demonstrated by flow cytometry with a rabbit antiserum raised to *in vivo* *L. mexicana* amastigotes. Amastigotes and promastigotes were labeled with either the pre-bleed normal rabbit serum (NRS), the immune serum, or with serum absorbed with $5 \times 10^7$, $5 \times 10^8$, or $5 \times 10^9$ promastigotes per ml (see Materials and Methods). The relative cell number is indicated on the y axis and the fluorescence is indicated on the x axis (log scale).
Figure 24  Antigenic differences between GP63 expressed in *L. mexicana* promastigotes and *in vitro* amastigotes

Lysates of *L. mexicana* promastigotes (Pr, lane 1) and *in vitro* amastigotes (Am, lane 2) were fractionated by denaturing SDS-PAGE and analyzed by duplicate western blot analysis using two different mAbs (mAb #235 and mAb #139) raised to *L. major* promastigote GP63. The lysates corresponded to 1.5 X 10^6 cells per sample.
(2) affinity purification of *in vitro* amastigote cell surface glycoproteins by concanavalin A-sepharose affinity chromatography

*L. mexicana* promastigote GP63 has been reported to contain high mannose N-linked carbohydrate groups with terminal mannosyl and glucopyranosyl residues (Olafson *et al.*, 1990). Therefore, concanavalin A (conA), which has a specificity for terminal α-D-mannosyl and α-D-glucosyl residues, was selected for purification of *Leishmania* N-linked glycoproteins (Reeke *et al.*, 1974). To demonstrate the efficacy of conA glycoprotein purification to enrich for cell surface proteins, GP63 was purified from lysates of *in vitro* amastigotes by passage over a conA-sepharose column as described in Materials and Methods. In Figure 25B, western blot analysis of total amastigote lysate (lane 1) and the conA column flow through (lane 2) and conA+ eluate (lane 3) fractions demonstrated efficient purification of GP63 (in unlabeled lysates) by conA affinity chromatography. Cell surface expression of GP63 on *in vitro* amastigotes was confirmed by flow cytometry using a rabbit antiserum raised against purified *L. major* rGP63 (Figure 25A). The shift in fluorescence was similar to that seen for *L. mexicana* promastigotes labeled with this serum (data not shown).

Lysates of amastigotes surface labeled with biotin were also passed over a conA-sepharose column as described in Materials and Methods. Biotin-labeled proteins were detected on blots with streptavidin-alkaline phosphatase and enrichment for GP63 was followed as a positive control. Analysis of the conA+ eluate from biotin labeled amastigotes (Figure 26) revealed the presence of a number of labeled proteins which may correspond to cell surface proteins. The identification of biotin labeled GP63 was confirmed by immunoprecipitation (data not shown). Other prominent bands of 35 kDa, 32 kDa, and 18 kDa were observed and are indicated in Figure 26. Relatively minor bands of 54 kDa, 47 kDa, 44 kDa, 24 kDa and 22 kDa were also seen (Figure 26). Silver stained SDS-PAGE of conA+ glycoproteins isolated from unlabeled amastigotes, revealed a pattern
Figure 25  Cell surface expression of GP63 on *in vitro* amastigotes and enrichment with conA

(A) Cell surface expression of GP63 on *in vitro* amastigotes was demonstrated by flow cytometry using rabbit #21 polyclonal antiserum raised against purified *L. major* GP63. Normal rabbit serum (NRS) was used as a negative control. Cells were labeled as described in Materials and Methods. The relative cell number is indicated on the y axis and the fluorescence is indicated on the x axis (log scale). (B) The potential of conA glycoprotein purification to enrich for cell surface molecules was demonstrated by the co-purification of GP63 in a fraction of glycoproteins purified by conA affinity chromatography. Co-purification of GP63 was demonstrated by western blot analysis of the column fractions: 1 μl of total amastigote lysate, 10^6 cells per ml (lane 1), 1 μl of conA− flow through (lane 2), aliquot of conA+ eluate. The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated on the left margin.
Figure 26 ConA affinity purification of biotin-labeled glycoproteins isolated from surface labeled in vitro amastigotes

Cell lysates of in vitro amastigotes, surface labeled by incubation with BiotinXNHS, were affinity purified over a conA sepharose column as described in Materials and Methods. Fractions of the total lysate (b-total, lane 1), the column flow through (b-flow through, lane 2) and the eluate (b-conA+ eluate, lane 3) were separated by SDS-PAGE (12% ), electroblotted, and developed with streptavidin alkaline phosphatase. The position and apparent molecular mass (kDa) of pre-stained protein standards is indicated in the left margin. The position and apparent molecular mass (kDa) of the major protein bands in the b-conA+ eluate is indicated in the right margin. The position of GP63 was confirmed by immunoprecipitation (data not shown).
similar to that of the biotinylated conA\(^+\) proteins (Figure 27, lane 3) suggesting that many of the eluted proteins may be surface molecules. Figures 25 and 26 therefore demonstrate the potential of con A purification for the isolation of amastigote surface glycoproteins. However, comparing the \(M_r\) of bands in the two preparations does not provide definitive evidence that a given unlabeled protein corresponds to a given biotin labeled protein. Moreover, a direct demonstration of the cellular localization of a given glycoprotein (e.g. by immunocytochemistry) would ultimately be required to confirm cell surface expression of any glycoprotein(s) for which the corresponding gene is cloned.

A fraction of the conA eluate was separated by SDS-PAGE and electroblotted onto ImmobilonP-SQ membrane, and stained with coomassie blue R250. The band corresponding to GP63 was then isolated and subjected to amino acid analysis to quantitate the amount of protein present. The yield of GP63 from an initial lysate of \(10^{10}\) cells was estimated at approximately 10 micrograms which corresponds to 159 picomoles. Therefore, the major bands in a preparation of \(10^{10}\) cells should be present in sufficient quantities for sequencing.

(3) \textbf{NH}_2-\textbf{Terminal amino acid sequence analysis of \textit{in vitro} amastigote glycoproteins}

The most prominent bands in the conA eluate, aside from GP63, were bands of approximately 30 kDa, 16 kDa and 13 kDa (Figure 27, lane 3). However, these bands were very similar in \(M_r\) to proteins detected in the supernatant fraction of reduced and boiled conA-sepharose beads (Figure 27, lane 4). Also, conA monomers have a \(M_r\) of 30 kDa (Reeke \textit{et al.}, 1974). Therefore, glycoproteins were also purified over a lentil lectin column and the eluted proteins compared to the conA eluate. Lentil lectin has the same sugar specificity as conA but has a monomeric \(M_r\) of 20 kDa (Howard \textit{et al.}, 1971). The prominent 30 kDa \(M_r\) band visible in the conA eluate (Figure 27, lane 3) was notably absent from the lentil lectin eluate (Figure 27, lane 7) suggesting that this band is likely
Figure 27  ConA and lentil lectin affinity purification of *in vitro* amastigote glycoproteins and further fractionation by differential EtOH precipitation

Glycoproteins were purified from *L. mexicana in vitro* amastigote lysates by passage over either a conA sepharose column (Sigma) or a lentil lectin sepharose column. The eluted material was further fractionated by precipitation with 77% EtOH and the samples were analyzed by silver stained SDS-PAGE (14%) (A) total lysate (lanes 1, 5), conA column flow through (lane 2), conA⁺ eluate (lane 3), boiled conA sepharose (lane 4), lentil lectin column flow through (lane 6), lentil lectin⁺ eluate (lane 7), boiled lentil lectin sepharose (lane 8), EtOH supernatant from conA⁺ eluate (lane 9), EtOH supernatant from lentil lectin⁺ eluate. Equivalent amounts of total and flow through samples were added in each lane. (B) EtOH precipitate from conA⁺ eluate (lane 1), EtOH precipitate from lentil lectin⁺ eluate (lane 2). The position and apparent molecular mass (kDa) of BioRAD low range protein standards is indicated on the left margin in (A). The position and apparent molecular mass (kDa) of protein bands isolated for NH₂-terminal sequence analysis (Tables 5 and 6) is indicated in (B).
conA. This was later confirmed by NH$_2$-terminal sequence analysis (Table 5). However, the 16 kDa and 13 kDa bands were present in both eluates. Analysis of boiled lentil lectin-sepharose beads is also shown (Figure 27, lane 8). The 20 kDa band present in the lentil lectin eluate is likely lentil lectin monomer. Apart from the differences noted above, the lentil lectin and conA column eluates appeared similar although there were qualitative differences in the relative proportions of some bands.

All of the major glycoproteins present in the conA$^+$ and lentil lectin$^+$ eluates were isolated for NH$_2$-terminal amino acid sequence analysis to obtain data for designing oligonucleotides primers. The eluates were concentrated and fractionated by precipitation with 77% EtOH, as described in Materials and Methods. The 16 kDa and 13 kDa bands were isolated in the EtOH supernatant (Figure 27A, lanes 9 & 10) with approximately 75% efficiency. However, sequence analysis of lyophilized EtOH supernatent was hindered by the presence of detergent and other contaminants which remained in this fraction. Therefore, both the EtOH supernatants and the EtOH precipitates were further fractionated by SDS-PAGE and electoblotted onto Immobilon-P-SQ membrane. Individual bands were then isolated from coomassie stained blots and subjected to NH$_2$-terminal amino acid sequence analysis. The samples analyzed corresponded to a starting eluate from 6 x 10$^9$ cells. The bands isolated from the EtOH precipitates for sequencing are indicated in Figure 27B. The 16 kDa band, indicated in Figure 27A, was isolated from the EtOH supernatent fraction.

The results of the NH$_2$-terminal sequence analyses are summarized in Tables 5 and 6. Each of these sequences was used to search the NCBI data base (Altschul et al., 1990) for similarity to reported sequences (translated from the DNA sequence). Putative identities were established for eight of the analyzed glycoproteins based on the sequence alignments shown in Figure 28. Sequences which did not match any reported Leishmania sequences were designated as "unique" in Tables 5 & 6. No sequence data was obtained for the 200 kDa, 35 kDa, and 25 kDa bands isolated from the conA eluate (Table 5) nor for
Table 5  Summary of NH$_2$-terminal amino acid sequence analysis of glycoproteins from *L. mexicana in vitro* amastigotes purified by conA lectin affinity chromatography and SDS-PAGE

<table>
<thead>
<tr>
<th>protein (kDa)*</th>
<th>sequence obtained **</th>
<th>putative identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>no sequence obtained</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>VASVPSDLYVLAWDT??Q</td>
<td>GP63 fragment</td>
</tr>
<tr>
<td>43</td>
<td>APPDAVDLVEK gva</td>
<td>Cys proteinase</td>
</tr>
<tr>
<td>35</td>
<td>no sequence obtained</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>GPEVFD?SPKRDQA ln y f r n</td>
<td>unique</td>
</tr>
<tr>
<td></td>
<td>ADTIVA??LD?YP?TD</td>
<td>conA</td>
</tr>
<tr>
<td>30</td>
<td>ADTIVAVELDTPNTDIGDPSYPKI</td>
<td>conA</td>
</tr>
<tr>
<td>25</td>
<td>no sequence obtained</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>APRPVMVEQVI?F?K s</td>
<td>Cys proteinase (fragment)</td>
</tr>
<tr>
<td>13</td>
<td>APRPV?VEQVII?DKNNRRG?RRT n r</td>
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</tr>
<tr>
<td></td>
<td>STHETNALHFMFNQFSKDKDLILQ</td>
<td>conA (fragment)</td>
</tr>
</tbody>
</table>

* these proteins are designated in Figure 27
** lower case letters indicate alternative amino acid assignments at positions of uncertainty
? mark indicates lack of assignment at a given position (may be a Cys residue)
Table 6 Summary of NH$_2$-terminal amino acid sequence analysis of glycoproteins from *L. mexicana* in vitro amastigotes purified by lentil lectin affinity chromatography and SDS-PAGE

<table>
<thead>
<tr>
<th>Protein (kDa)*</th>
<th>Sequence obtained **</th>
<th>Putative identity</th>
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</thead>
<tbody>
<tr>
<td>51 GTEGA</td>
<td>unknown ***</td>
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</tr>
<tr>
<td>43 APPDAVETRLK</td>
<td>Cys proteinase</td>
<td>unique</td>
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<tr>
<td>ytvqp tyv</td>
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<td></td>
</tr>
<tr>
<td>37 ANPLFVR</td>
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<tr>
<td>vltld q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 no sequence obtained</td>
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<td>33 APTSTRM</td>
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<tr>
<td>29K AEDDE</td>
<td>unknown ***</td>
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<tr>
<td>dp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* these proteins are designated in Figure 27

** lower case letters indicate alternative amino acid assignments at positions of uncertainty

? mark indicates lack of assignment at a given position (may be a Cys residue)

*** amino acid assignment extremely uncertain; only femtomole quantities obtained
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence (NH2-terminal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 48kDa protein</td>
<td>VASVPSDLVYVLAWDT??Q</td>
</tr>
<tr>
<td>L. mexicana GP63c1</td>
<td>216 VASVPSEESVLAWATTCQ 233</td>
</tr>
<tr>
<td>B. 43kDa protein</td>
<td>AVPDAVDLVEK</td>
</tr>
<tr>
<td>L. mexicana lmcpb Cys proteinase</td>
<td>125 AVPDAVDWREK 135</td>
</tr>
<tr>
<td>C. 30kDa protein</td>
<td>ADTIVAVELDTYPNDIGPSYPKI</td>
</tr>
<tr>
<td>Canavalia ensiformis conA</td>
<td>1 ADTIVAVELDPNTDIDGPSYPHI 25</td>
</tr>
<tr>
<td>D. 16kDa/13kDa proteins</td>
<td>APRPVMEQVIVIFDKNNRG?RRT</td>
</tr>
<tr>
<td>L. mexicana lmcpb Cys proteinase</td>
<td>356 APRPVVMEQVICFDKNCRRGCRKT 380</td>
</tr>
<tr>
<td>L. pifanoi cys 2 Cys proteinase</td>
<td>356 APRPVVMEQVICFDKNCTQGCRKT 380</td>
</tr>
<tr>
<td>E. 13kDa protein</td>
<td>STHETNALHMNFQFSKDXDLILQ</td>
</tr>
<tr>
<td>Canavalia ensiformis conA</td>
<td>119 STHETNALHMNFQFSKDXDLILQ 143</td>
</tr>
</tbody>
</table>

**Figure 28** Protein sequence alignments of amastigote glycoprotein NH2-terminal sequences with published sequences

The NH2-terminal amino acid sequences reported in Tables 5 and 6 were submitted to BLAST-P to search for similar sequences in the database. The amastigote sequences are aligned with segments of similar published sequences. Sequence identity is indicated by double dots, a conservative amino acid substitution is indicated by a single dot, and lack of identity is indicated by a blank. The first and last amino acid of each published sequence is identified by number according to the reported sequences. (A) Medina-Acosta et al, 1993 (B) Souza et al, 1992 (C) Carrington et al, 1985 (D) Souza et al, 1992; Traub-Cseko et al, 1993 (E) Carrington et al, 1985
the 36 kDa band isolated from the lentil lectin eluate (Table 6). Based on the poor yields observed for the lentil lectin bands analyzed (Table 6), the 45 kDa band from this eluate and the 80 kDa band from the conA eluate, were not sequenced.

**B. Discussion**

The *in vitro* amastigote model was shown to provide a feasible approach for isolation of microgram quantities of amastigote glycoproteins using conA and lentil lectin affinity chromatography (Figures 23-25, 27). With this approach large numbers of amastigote-like cells are readily obtained and the difficulties involved in purifying lesion amastigotes from macrophages are avoided. The similarity of conA+ glycoproteins isolated from unlabeled amastigotes and biotin surface labeled amastigotes suggests that many of the purified glycoproteins were surface proteins (Figures 26-27). Furthermore, the preparation of samples for sequencing was very effective as sequence data was obtained for 12 of the 15 bands analyzed (Tables 5 and 6). Of the 12 sequences obtained, 5 appear to be unique. However, analysis of larger quantities of these proteins is needed to obtain more reliable and longer sequences.

The 43 kDa band in the conA and lentil lectin eluates appears to be a Cys proteinase encoded by the *lmcpb* gene family; the sequence of the 43 kDa band aligns with the mature NH2-terminus observed for purified Cys proteinase (Robertson and Coombs, 1994). Although there were several uncertainties in the sequence of the 43 kDa band from the lentil lectin eluate (Table 6), the sequence AxPDAVxLVxK was confirmed in the sequence obtained for the 43 kDa band from the conA eluate (Table 5). The *lmcpb* gene family consists of 10 or more tandem repeats of this gene (Souza *et al.*, 1992) and appears to encode at least three groups of Cys proteinases (A, B and C) with differing substrate specificities and physical properties (Robertson and Coombs, 1990 and 1994). Different
lmcpb genes within the family are thought to encode limited amino acid substitutions accounting for the different properties of the mature enzymes. A homologous multi-gene family, lpcys2, has also been identified in L. pifanoi and has extensive sequence similarity with the lmcpb gene (Traub-Cseko et al., 1993). The Cys proteinases are expressed at high levels in the megasomes of L. mexicana and L. pifanoi amastigotes and may constitute up to 1% of the total cellular protein (Pupkis et al., 1986; Traub-Cseko et al., 1993). NH2-terminal sequences determined for group A, B and C lmcpb Cys proteinases are all highly similar and closely match the predicted sequence from the cloned lmcpb gene (Robertson and Coombs, 1994). Whereas the group B and C L. mexicana Cys proteinases do not appear to be glycosylated, the group A Cys proteinases are N-glycosylated and bind conA (Robertson and Coombs, 1990). Therefore, the 43 kDa glycoprotein is likely a group A Cys proteinase.

The lmcpb proteinases appear to be encoded as prepro enzymes (Souza et al., 1992) (Figure 29A). Comparison of the cloned lmcpb gene sequence to other trypansomatid Cys proteinases predicts that the mature enzymes contain a central domain of 218 aa and a COOH-terminal (C-terminal) extension of 100 aa (Aslund et al., 1991; Souza et al., 1992). The predicted molecular weight of the mature Cys proteinases is 34 kDa (without carbohydrate). However, purified group B and C enzymes, and deglycosylated group A enzymes have a reported Mr of 24 kDa which closely approximates the predicted Mr of the central domain (23.6 kDa, Robertson and Coombs, 1994). Robertson and Coombs (1994) propose that the C-terminal extension is cleaved autolytically between Pro-355 and Ala-356 (Figure 29B). As shown in Figure 28, the sequence obtained for the 16 kDa and 13 kDa bands aligns with amino acids 356-380 of the lmcpb and lpcys 2 Cys proteinases. Thus, the 16 kDa and 13 kDa bands appear to correspond to the predicted 83 amino acid fragment of the C-terminal extension (Figure 29D). The 16 kDa and 13 kDa glycoproteins are therefore predicted to contain approximately 7 kDa and 4 kDa of carbohydrate, respectively. Although the cloned lmcpb gene does not contain a predicted N-linked
Figure 29 Schematic diagram of *L. mexicana* Cys proteinase and the 43kDa, 16 kDa and 13 kDa glycoproteins purified from *L. mexicana in vitro* amastigotes.

The predicted prepro-precursor Cys proteinase (A) and mature Cys proteinase (B) are shown (Souza et al, 1992). The predicted N-linked glycosylation site in the central domain (N228) is indicated (CHO=carbohydrate). The predicted N-linked glycosylation site in the C-terminal extension (N371) of the *L.pifanoi* cys 2 proteinase is also indicated (Traub-Cseko, 1993). The putative structures of the 43 kDa (C) and the 16kDa and 13KDa (D) *L. mexicana* amastigote glycoproteins are also shown. Amino acids are numbered according to Souza et al (1992).
glycosylation site within the C-terminal extension, the cloned lpcys2 gene has a predicted glycosylation site at Asn-371. Perhaps the 16 kDa and 13 kDa bands are fragments of different group A isoforms with different amounts of carbohydrate.

The 43 kDa glycoprotein isolated from the conA and lentil lectin eluates has a significantly higher $M_r$ than the reported $M_r$ of 28 kDa for mature group A Cys proteinases. However, adding the observed $M_r$ of the 16 kDa glycoprotein, corresponding to the C-terminal extension, predicts a $M_r$ of 44 kDa. Thus, the 43 kDa glycoprotein appears to be immature group A proteinase containing the C-terminal extension (Figure 29C).

In addition to the sequences identified as GP63, conA and Cys proteinases (Table 5 and 6), 5 unique sequences were also found. However, as indicated in Table 6, the amino acid assignments are very tentative for proteins in the lentil lectin eluate because only femtomole yields were obtained on the sequencer. By analyzing larger quantities of each of these proteins, more reliable and longer sequences should be readily attained. Two sequences were obtained for the 34 kDa band in the conA eluate. Although one sequence corresponds to the NH$_2$-terminal sequence of conA, the second sequence appears to be unique and is long enough to design a probe or primer (Sambrook et al., 1989). However, because of the uncertainties at several residues the sequence should be confirmed before proceeding further.

Once more reliable protein sequence data is obtained and confirmed to be unique, the following strategy could be employed to clone the corresponding gene(s) and provide a preliminary characterization:

1) design oligonucleotide primers to amplify cDNA fragments from in vivo amastigote cDNA using the strategy outlined in the Appendix.

2) clone and sequence the PCR products to identify the correct cDNA fragment

3) use the cDNA fragment as a probe to screen a genomic library to isolate a full length clone
4) determine if expression is stage-specific by northern blot analysis of amastigote and promastigote RNA
5) sequence the gene and look for similarity to reported sequences
6) analyze the predicted protein sequence for potential transmembrane or GPI signal sequences
7) determine the cellular localization by immunochemistry with antibodies raised to recombinant protein.

Although a strategy was chosen to target cell surface proteins, a direct demonstration of cell surface expression, as outline above, would be critical for any genes cloned by this approach. The similarity of the pattern of surface biotinylated and unlabeled conA+ glycoproteins (Figures 26 & 27) is not direct evidence of cell surface expression. Furthermore, in comparing Figures 26 and 27 it appears that bands corresponding to the 43 kDa, 16 kDa and 13 kDa bands identified as Cys proteinases, may have been biotinylated. Although there is evidence that cell surface Lmcpb products may exist (Robertson and Coombs, 1994), groups A,B and C Cys proteinases are not expressed on the cell surface but are localized to the megasomes, which are thought to represent the lysosomal compartment in *Leishmania*. Perhaps biotin was endocytosed at the flagellar pocket and gained access to the megasome compartment by endocytic vesicle-megasome fusion (Webster and Russell, 1993).

In conclusion, this study demonstrates that the most abundant conA+ and lentil lectin+ *L. mexicana* amastigote glycoproteins are Cys proteinases, GP63, and fragments thereof. However, a number of unique glycoproteins are also present and warrant further study.
Chapter 5. General Discussion

Proteinases have been implicated in the pathogenesis of protozoan parasites in a variety of roles including invasion of host tissues, degradation of host immune defense mediators such as complement, and evasion of macrophage-mediated killing by degradation of lysosomal enzymes (reviewed by North et al., 1990; Horstmann et al., 1992; McKerrow et al., 1993). In Chapter 4, the most abundant conA+ glycoproteins in *L. mexicana* in vitro amastigotes were shown to consist of GP63 proteinase and Cys proteinases which *in vivo* are expressed at high levels in the amastigote megasome compartment (Robertson and Coombs, 1990). In the promastigote, GP63 is the major surface glycoprotein and constitutes 1% of the total cellular protein (Bahr et al., 1993). Cell surface GP63 appears to be down-regulated in amastigotes, and a stage-specific GP63 localized to the megasome compartment has been reported (Bahr et al., 1993). Although the function of *Leishmania* GP63 and Cys proteinases has yet to be determined, the abundance of these proteinases suggests their importance in the parasite life cycle.

Aside from evidence that GP63 mediates uptake of *Leishmania* by macrophage cells and may participate in evasion of innate host defenses (see Chapter 1), very little is known about the function of this proteinase in either the promastigote or amastigote life stages. The characterization of GP63 as a matrix metalloproteinase in Chapter 3, however, suggests that GP63 could be involved with the pathogenesis of cutaneous lesion formation and metastasis within the mammalian host. The involvement of mammalian MMPs in tumour invasion and metastasis has been well established (reviewed by Mignatti and Rifkin, 1993). As discussed in Chapter 1, the MMPs are one component of a complex proteolytic cascade which mediates matrix degradation.

The recently identified MT-MMP (Sato et al., 1994) may have a role similar to uPA in initiating activation of ECM degradation (Vassalli and Pepper, 1994). Cell surface MT-MMP was shown to convert pro-gelatinase A (MMP-2) to its active form; additional
substrates for MT-MMP remain to be determined however. Perhaps GP63 expressed on the surface of infective promastigotes acts in a manner analogous to MT-MMP or to uPA, initiating activation of the host proteolytic cascade and thereby mediating local tissue destruction by degradation of the ECM. This in turn would enhance the local inflammatory response and infiltration of macrophages which could then be parasitized. However, extracellular promastigotes are present only briefly in the initial stage of infection. Thus, any role that GP63 might have in degradation of the ECM may be most important in the persistent amastigote stage mediating local tissue invasion and contributing to the pathology of lesion formation which is thought to be largely immune-mediated by a chronic inflammatory response resulting in local ECM degradation and remodelling (Andrade et al., 1984; Esterre, et al., 1991; Pirmez, 1992). Similarly, perhaps amastigote GP63 has a role in metastasis observed in disseminating forms of leishmaniasis (i.e. diffuse cutaneous, mucocutaneous, and visceral leishmaniasis).

In order to investigate these potential roles for GP63 in degradation of the host ECM, several questions must be addressed. Can GP63 activate pro-uPA, plasminogen, or pro-gelatinase A and other proMMPs? Does expression of rGP63 on the surface of transfected cells increase their invasive potential as measured in standard tumour invasion assays? In addition, a detailed analysis of the substrate specificity of GP63 for ECM components would also be instructive. Ultimately, the generation of Leishmania GP63 deletion mutants and mutants expressing an enzymatically inactive GP63 gene (e.g. GP63-ASP265) may provide key tools permitting a direct examination of the in vivo role of GP63 in different life stages, both within the mammalian host and the insect vector.

It has been shown that L. major infections in the sandfly Phlebotomus papatasi lead to damage of the insect's main feeding valve and this seems to enhance transmission of the parasites by bite (Schlein et al., 1991 and 1992). The feeding mechanism is damaged by degradation of the cuticle lining and subsequent breakdown of the valve tissues. Cuticle degradation appears to be mediated by promastigote chitanases secreted in the insect foregut
but proteolytic enzymes are also thought to contribute to breakdown of the valve tissues. Perhaps GP63 may be involved and thereby contribute to parasite transmission to the vertebrate host. Moreover, GP63 may have a role in parasite metabolism.

Thus, GP63 proteinase activity may have distinct roles in different life stages of the parasite. Promastigote GP63 may contribute to parasite transmission in the sandfly and to establishment of infection in the mammalian host whereas amastigote GP63 may contribute to persistence of the parasite and metastasis. An understanding of the function of GP63 and other proteinases in the life cycle of *Leishmania* may provide valuable insights for the development of chemotherapeutic agents for treatment of Leishmaniasis and will contribute greatly to our understanding of the biology of the parasite.
Appendix: RT-PCR Generation of Amastigote cDNA and PCR Amplification Using a Primer Specific for the 43 kDa Glycoprotein

A. Experimental Procedures

(1) Purification of total RNA from lesion amastigotes and DNase I treatment
RNase free dH2O and other solutions were prepared by the addition of 0.1% DEPC and incubation for 15 m at room temperature prior to autoclaving. RNase free Tris buffers were prepared by dissolving Tris in DEPC-dH2O (pre-treated as above) and then autoclaving.

_L. mexicana_ lesion amastigotes were isolated and purified over cotton wool and the total RNA extracted using a single-step acid-guanidinium thioctyanate-phenol-chloroform extraction method (Chomcynski and Sacchi, 1987). Amastigotes were resuspended in the guanidinium thioctyanate denaturing solution and the RNA isolated as described (Chomcynski and Sacchi, 1987), substituting 95% EtOH for isopropanal and repeating the initial precipitation. The final RNA pellet was resuspended in DEPC-dH2O.

To eliminate contaminating DNA, total RNA was treated with DNase I (Pharmacia). Approximately 20 μg of total RNA was incubated for 1 hr at 37°C in the presence of 20 units DNaseI, 2 mM MgCl2, and 80 units RNasin (BRL) in a final volume of 50 μl. The reaction was stopped by the addition of 5 μl 50 mM EDTA and incubating for 5 min at 90°C. The RNA was then purified by phenol-chloroform extraction followed by EtOH precipitation. The final pellet was resuspended in 20 μl DEPC-dH2O.

(2) cDNA synthesis: Reverse Transcription and PCR

_L. mexicana_ amastigote cDNA was generated using RT-PCR; reverse transcription with a modified oligo-dT primer was followed by PCR amplification to generate double stranded cDNA (Figure 30). First strand cDNA synthesis was carried out using DNase I treated total RNA (from
Figure 30  Primer strategy for RT-PCR generation of an amastigote cDNA library

An oligo dT primer (3' mmRT) encoding a tail with XbaI and NotI restriction sites was designed to prime reverse transcription of polyA mRNA. A second set of primers was designed to amplify the first strand cDNA using PCR. A 5' PCR primer (5'SL) containing the splice leader sequence present at the 5' end of all Leishmania mRNAs and an EcoRI restriction site was designed. A 3' PCR primer (3'mm) containing the XbaI/NotI tail encoded by the 3'mmRT primer was also designed. This strategy should produce full length cDNA with unique restriction sites for cloning.

A.

\[
\begin{array}{c}
\text{SL} \\
\text{coding region} \\
\text{polyA}
\end{array}
\]

\[\text{**5'SL} \rightarrow \text{**3'mmRT} \rightarrow \text{**3'mm}\]

B. Primer Sequences

Reverse Transcription Primer (3'mmRT): 3' (T)17 AGATCTCGCCGGCGGAGG
XbaI Not I

3' PCR Primer (3'mm) 3' AGATCTCGCCGGCGGAGG
XbaI Not I

5' PCR Primer (5'SL): 5' GCCAGAATTCCAG TTT CTG TAC TTT ATT G
EcoRI

C. Splice leader sequence data used in design of the 5' SL PCR primer:

\begin{itemize}
  \item \textit{L. enriettii}: \texttt{A Acg cta Tat aag tat} CAG TTT CTG TAC TTt ATT G
  \item \textit{L. collosoma}: \texttt{aaa Aca att Ttt gaa gaa} CAG TTT CTG TAC TTt ATT G
  \item \textit{L. amazonensis}: \texttt{CAG TTT CTG TAC TTt ATT G}
\end{itemize}

lesion amastigotes) as a template for reverse transcription with the 3' RT-M primer, GGAGGCCGCGCTCTAGAGTTTTTTTTTTTTTTTTTTT. This primer introduces both an XbaI (underlined) and a NotI (double underlined) restriction site and contains a 17 n stretch of dT for priming polyA RNA. The BRL SuperScript RTaseH reverse transcriptase was used according to conditions described by the manufacturer in the preamplification system manual (BRL Cat No. 8089SA); nucleotides were from Cetus. Briefly, 3 μg amastigote RNA was incubated with 75 pmol primer, in a final volume of 15 μl for 10 min at 70°C and then quick chilled on ice. The entire volume was then mixed 1:1 with a reaction premix to give a final reaction of 30 μl containing 300 units SuperScriptase, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 μCi/μl alpha-32P-dCTP, 10 mM DTT, 20 mM TrisHCl pH 8.4, 50 mM KCl, 2.5 M MgCl2, 0.1 mg/ml BSA. After reverse transcription the reactions were digested with 2 units RNaseH (BRL) for 20 min at 37°C, diluted 2 fold with DEPC-dH20 and then 1 μl removed for determination of the total CPM. The remainder was purified by phenol/chloroform extraction followed by EtOH precipitation. The final pellet was resuspended in 32 μl DEPC-dH20 and 2 μl was removed for autoradiography and TCA precipitation (to quantitate 1st strand synthesis). Of the remainder, 20 μl was used as a template for PCR amplification to generate double stranded cDNA using the 5' SL primer and the 3' M primer. The 5' SL primer, GCCAGAAATCCCAGTTTCGTACTTTATTG, is complementary to the Leishmania RNA splice leader sequence (underlined) and also introduces an EcoRI restriction site (double underlined). The 3' M primer, GGAGGCCGCGCTCTAGA, is identical to the 5' portion of the 3' RT-M primer introducing both XbaI and NotI restriction sites. The 20 μl of template was added to 80 μl of a reaction premix and then divided into two 50 μl samples for PCR. The final reaction contained 1.25 units Taq (Cetus), 1x reaction buffer (Cetus), 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, 10% (vol/vol) DMSO and 0.2 μCi/μl alpha-32P-dCTP. Amplification was performed in a Perkin Elmer Cetus GeneAmp 9600 thermocycler with 30 cycles of [94°C, 30 s; 45°C, 1 m; 72°C, 4 m + 5 s auto-extend] followed by a final extension step of 72°C, 10 min. Control reactions included the following: no reverse transcriptase, to test for
contaminating DNA at the PCR amplification step; no RNA, to test for contaminating template in the reagents; and irrelevant RNA, to test for specific amplification of *Leishmania* RNA by the 5' PCR primer. The irrelevant RNA was isolated from a murine T-cell hybridoma.

**B. Results**

RT-PCR was used to generate *L. mexicana* in vivo amastigote cDNA as shown in Figure 30. An oligo-dT primer (3'mmRT) was designed to prime reverse transcription of total RNA isolated from *in vivo* grown *L. mexicana* lesion amastigotes. The first strand *Leishmania* cDNA was then amplified by PCR using a 5' primer (5'SL) specific for the splice leader sequence (Figure 30C) found on all *Leishmania* mRNAs (Miller et al., 1986) and a 3' primer (3'mm) specific for a cloning site introduced by the reverse transcription primer. The reverse transcription (first strand synthesis) and PCR (second strand synthesis) reactions were monitored by autoradiography of an agarose gel of reactions containing 32P-dCTP (Figure 31). The 5'SL primer strategy eliminated the problem of contaminating murine RNA likely present in the template amastigote RNA. To demonstrate this, RNA isolated from a murine T-cell hybridoma was used as a negative control (Figure 31, lane 3). As expected, first strand cDNA was generated in reverse transcription reactions using either murine RNA (Figure 31A, lane 3) or amastigote RNA (Figure 31A, lane 4) as a template. However, the second strand synthesis was specific for *Leishmania* cDNA (Figure 31B, lane 4); no PCR product was detected for the murine RNA control (Figure 31B, lane 3). Controls with either no RNA template (Figure 31, lane 1) or no reverse transcriptase (Figure 31, lane 2) were negative in both reactions. Starting with 1 µg of total amastigote RNA as a template, approximately 3.7 mg of amastigote cDNA was generated. To examine the integrity of the RT-PCR cDNA, an aliquot of cDNA was double digested with XbaI/EcoRI and cloned into the pAX82 vector, a derivative of pAX111. Based on analysis of test transformations, the ligated cDNA contained ≥ 75%
RT-PCR was used to generate cDNA from RNA isolated from *L. mexicana* lesion amastigotes. RNA from a murine cell line was used as a control for amplification of contaminating murine RNA template. $^{32}$P-dCTP was added to the reverse transcription reactions (A) and the PCR amplifications (B) and the products were separated by agarose gel electrophoresis and visualized on an autoradiogram. Reverse transcription was carried out using the 3'mmRT primer shown in Figure 30 and PCR was carried out using the 5'SL and 3'mm PCR primers depicted in Figure 30. The RNA templates in the reverse transcription reactions in (A) were as follows: no RNA (lane 1), *L. mexicana* amastigote RNA (lanes 2, 4), and murine T cell hybridoma RNA (lane 3). No reverse transcriptase was added in lane 2. Following reverse transcription, and aliquot of each of the reactions in (A) was used as a template for PCR in (B). The position and size (kb) of DNA size markers is indicated on the left margin in (A) and (B).
recombinants with varying insert size ranging from 0.5 kb - 1.8 kb (data not shown). Promastigote cDNA was also generated using the RT-PCR method described above.

Based on the sequence data obtained for the 43 kDa glycoprotein (Table 5), a 3' oligonucleotide PCR primer (MH43K) was designed to be used with the 5'SL primer to amplify a 5' cDNA fragment from the in vivo amastigote cDNA (Figure 32). The DNA sequence was predicted based on the strict codon usage reported for Leishmania (Langford et al., 1992). A BamH1 restriction site was incorporated into the MH43K primer to facilitate sub-cloning of the PCR products. As shown in Figure 32, the MH43K primer is predicted to amplify a 581 bp cDNA fragment of the lmcpb gene. Figure 33 shows the PCR fragments generated by amplification of amastigote cDNA (lane 3) and promastigote cDNA (lane 4). At least six different bands were generated, one of which corresponds to the predicted size and therefore may represent a Cys proteinase cDNA fragment.

If the 43 kDa protein is not a member of the Cys proteinase family, the other PCR products may correspond to unique cDNAs. The prominent 800 bp band was purified from an agarose gel slice, digested with BamH1, and sub-cloned into bluescript (Stratgene). Two different subclones of 800 and 700 bp were subsequently isolated for DNA sequence analysis. Both clones contained the 5'SL and MH43K primer sequences. However, the DNA sequence immediately upstream of the MH43K primer sequence did not correspond to the predicted DNA sequence (Figure 32). Thus, neither subclone corresponded to the 43 kDa glycoprotein.
A.

1. Ala Pro Pro Asp Ala Val Asp Leu Val Glu Lys
2. GCg CCg CCg GAc GCg GTg GAc CTg GTg GAg AAg
3. Gly Val Ala
4. GGC GTg GCg

B.

1. GTg GAc CTg GTg GAg
2. MH43K 3' primer 3' CAC CTG GAC CAC CTA GCGG 5'

**Figure 32** Sequence and design of MH43K 3' primer for PCR amplification of a cDNA fragment encoding a 43 kDa conA+ amastigote glycoprotein

(A) The DNA sequence (line 2) encoding the NH2-terminal amino acid sequence (line 1) obtained for the 43 kDa glycoprotein was predicted based on the reported codon usage for *Leishmania* (Langford et al, 1992). Nucleotides given in lower case letters indicate uncertainty due to the redundancy of the genetic code. The amino acid residues and corresponding DNA sequence given in lines 3 and 4 represent alternative amino acid assignments at these positions. The underlined sequence was used to design the MH43K primer. (B) The MH43K 3' primer (line 2) is complementary to the coding strand (line 1) and was designed to incorporate a BamHI restriction site. The nucleotides indicated in bold are not contained within the amino acid sequence data. This primer was designed to use in conjunction with the 5'SL primer to amplify a 5' cDNA fragment using amastigote cDNA as a template.
Figure 33  DNA fragments obtained by PCR amplification of amastigote and promastigote cDNA using the 5'SL primer and the 3'MH43K primer

PCR reactions with the 5'SL primer and the 3'MH43K primers were performed using either 50 ng of in vivo amastigote cDNA (lane 3, Am) or 50 ng of promastigote cDNA (lane 4, Pr) as a template. The reactions were EtOH precipitated and then analyzed by agarose gel electrophoresis in the presence of ethidium bromide. The Pharmacia 100 bp ladder (lane 1) and the BRL 1 kb ladder (lane 2) were included as DNA size markers.
References


Bode, W., Gomis-Ruth, F.X and Stockler, W. (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett.* 331(1,2):134-140.


