STRUCTURE AND EXPRESSION OF GP63 GENES IN *LEISHMANIA MAJOR*

by

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ABSTRACT

Leishmania are protozoan pathogens responsible for the human disease known as leishmaniasis. *Leishmania* parasites alternate between an insect promastigote stage and an amastigote stage found in infected macrophage cells of mammalian hosts. The major surface protein found on promastigotes is a glycoprotein with a molecular weight of 63 kDa and is referred to as GP63. Restriction map analysis of genes encoding GP63 in *Leishmania major* indicate that GP63 is encoded by five conserved tandemly repeated genes and a less conserved sixth gene separated from the five repeats. In this study, DNA sequence analysis of the sixth gene of the *L. major* gp63 locus indicated that this gene encodes a different form of GP63 with an extended carboxy terminus in comparison to GP63 encoded by gene one[1]. Northern blot analysis of gp63 expression in *L. major* demonstrated that *L. major gp63-1* is expressed in the promastigote stage whereas *L. major gp63-6* is expressed in both the promastigote and intracellular amastigote stage. Furthermore, *L. donovani* promastigotes transfected with *L. major gp63-6* were shown to express GP63 as a surface protein in FACScan analysis using an *L. major* specific anti-GP63 antibody suggesting that *L. major* GP63-6 is expressed as surface protein in promastigotes.

[1]*L. major* gp63 gene 6 will be referred to as *L. major gp63-6* hereafter with lowercase italicized *gp63-6* representing the DNA. Uppercase unitalicized GP63-6 will refer to the protein. Likewise, *L. major* gp63 gene 1 will be referred to as *L. major gp63-1* while the protein will be represented by *L. major* GP63-1.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
BSA	bovine serum albumin
cpm	counts per minute
dATP	deoxyriboadenosine 5' triphosphate
dCTP	deoxyribocytidine 5' triphosphate
DMEM	Dulbecco's modified eagle media
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucloside 5' triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
FACScan	fluorescence activated cell scanning
FCS	fetal calf serum
HEPES	4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid
kb	kilobase pair(s)
kDa	kilodalton(s)
LB	Luria-Bertani
MOPS	3-(N-morpholino)-propanesulfonic acid
NBT	nitroblue tetrazolium chloride
PCR	polymerase chain reaction
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
Tris	tris(hydroxymethyl)aminomethane
U	units

WHO	World Health Organization

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I. INTRODUCTION

A. AN OVERVIEW OF LEISHMANIASIS

Leishmania are clinically important protozoan parasites responsible for a complex and diverse human disease known as leishmaniasis. *Leishmania* parasites are dimorphic pathogens that live in two distinct environments, alternating between a blood-sucking sandfly vector and mammalian macrophage cells of the mononuclear phagocyte lineage.

It has been estimated that there are at least 12 million cases of leishmaniasis in over 80 countries with 350 million people at risk. The disease is found predominantly in countries near the equator (Walsh and Warren, 1979). Restriction of leishmaniasis to tropical and sub-tropical regions is largely the result of the breeding range of the sandfly vector.

At least 14 different species and subspecies of *Leishmania* are responsible for human leishmaniasis (Wirth *et al.* 1986). Clinically, leishmaniasis is found to exhibit a diverse array of symptoms with the disease type depending on the species of infecting *Leishmania*. The broad range of manifestations of the disease can be characterized into three main clinical forms: simple cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis (for a review of leishmaniasis, see Chang *et al.* 1985). Simple cutaneous disease is characterized by skin lesions and ulcers as a result of an immune response to parasitized macrophages at the surface of the skin. This form of the disease is often self limiting with lesions healing after a few months. Mucocutaneous leishmaniasis often begins as a simple cutaneous lesion with subsequent metastasis of infected macrophages to the nasal mucosa with resulting tissue destruction of the nose and mouth. Visceral leishmaniasis (also known as Kala-Azar) is the most serious form of the disease with infected macrophages of the spleen, liver, lymph nodes, as well as the skin. Clinically, visceral leishmaniasis is characterized by fever, malaise, weight loss, anemia, and

immunosuppression. Immunosuppression ultimately leads to other infections and death if untreated. Table 1 lists several species of *Leishmania* and the form of the disease they cause.

The most common form of treatment for human leishmaniasis is a long course of daily injections of pentavalent antimony (for a review on treatments of leishmaniasis, see Olliaro and Bryceson, 1993). This form of chemotherapy is expensive with possible side effects. Recent reports have shown that drug resistant strains are becoming more prevalent. It has also been reported that there is a 30% relapse rate after treatment with antimonials (Olliaro and Bryceson, 1993).

The first recombinant antigen to be used in vaccine trials was the major promastigote surface glycoprotein of 63 kD molecular weight referred to as GP63. (GP63 has also been referred to as promastigote surface protease or PSP). GP63 has been shown to induce some level of protection in mice (Russell and Alexander, 1988; Yang *et al.* 1990, Jardim *et al.* 1990). More recently, immunization of CBA/J mice with recombinant *bacille Calmette-Guérin* expressing GP63 was shown to provide significant protection against infectious *Leishmania* (Connell *et al.* 1993).

Table 1. Major *Leishmania* species causing human disease.

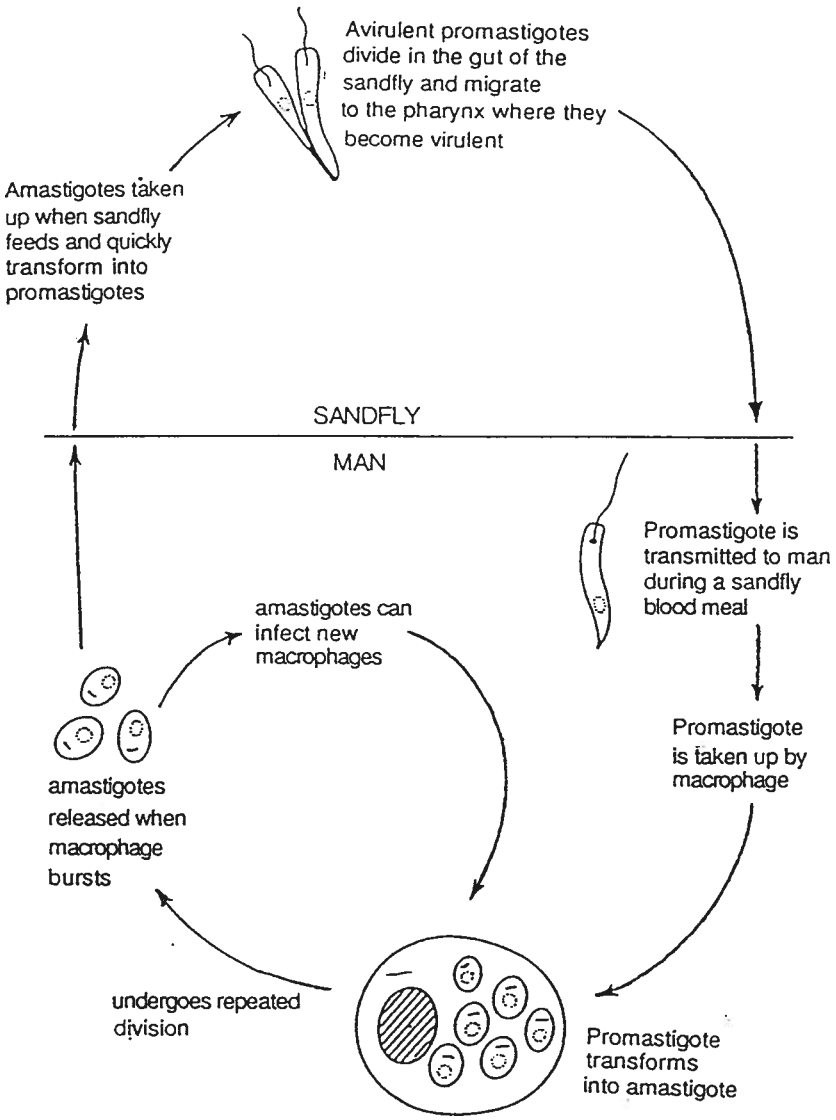
Disease	Species	Geographical Location
Cutaneous leishmaniasis		
Simple cutaneous leishmaniasis	L. mexicana mexicana	Mexico, Central America
	L. mexicana amazonensis	Brazil, Amazon region
	L. mexicana pifanoi	Venezuela
	L. major	Southern U.S.S.R., Middle East
	L. tropica	Asia, Southern Europe, Northern and western Africa
	L. braziliensis guyanensis	North and South America
	L. braziliensis panamensis	Central America
	L. braziliensis peruviana	Peru
Diffuse cutaneous disease	L. mexicana amazonensis	Brazil, Amazon region
	L. aethiopica	Ethiopia and Kenya
Mucocutaneous disease	L. braziliensis braziliensis	Western and northern South America
Visceral leishmaniasis		
	L. donovani	India, Africa
	L. donovani infantum	Mediterranean area
	L. chagasi	Northern, South America

Source of data: Wirth et al., (1986).

B. THE LIFE CYCLE OF LEISHMANIA

Members of the *Leishmania* genus undergo two distinct morphological stages in their life cycle. *Leishmania* exists as a flagellated promastigote in the gut of a sandfly vector of the subfamily Phlebotominae. Promastigotes divide by binary fission in the midgut of the insect and are avirulent at this point. As a result of nutrient depletion, promastigotes reach the stationary phase of growth and begin to migrate to the pharynx where they become virulent. Infectious or virulent forms of promastigotes are referred to as metacyclics (Sacks and Perkins, 1984). Transmission of the parasites to the mammalian host occurs when an infected sandfly takes a blood meal. Inside the blood stream of the mammalian host, promastigotes are taken up in a phagosome by macrophage cells via the process of phagocytosis. The phagosome becomes fused with host lysosomes inside macrophage cells forming a phagolysosome which is normally responsible for digestion and removal of foreign substances taken up by macrophage cells. Inside the phagolysosome, promastigotes transform into nonflagellated amastigotes and divide to infect more macrophage cells. Thus, *Leishmania* is able to survive and thrive in a cell and organelle which assists in clearing the body of invading organisms. Uptake of infected blood by a sandfly completes the cycle with the amastigotes reverting back to the promastigote form inside the gut of the insect vector. In addition to infecting humans, *Leishmania* infects other mammals including rodents and dogs which serve as significant reservoirs for the parasites (Chang *et al.* 1985). Figure 1 depicts the two stage life cycle of *Leishmania.*

Figure 1. The life cycle of *Leishmania*.



C. CHARACTERISTICS OF KINETOPLASTID GENE EXPRESSION

Leishmania belong to the Trypanosomatidae family and are members of the order Kinetoplastidia. Members of this order possess a unique organelle called the kinetoplast which is the single mitochondrion present in these protozoa (for reviews see Simpson, 1987; Ryan *et al.* 1988; Stuart and Feagin, 1992). *Leishmania* along with other trypanosomatid protozoan parasites are also unique in the organization and expression of their genetic information in several respects including the processing of mRNA by *trans*-splicing and RNA editing.

Highly expressed proteins in Kinetoplastid protozoa are often found to be encoded by multiple gene copies which are arranged in tandem direct repeat units often located at a single chromosomal locus (Van der Ploeg, 1986). Examples of multiple gene copies linked in a head to tail fashion include the mini-exon genes in *Trypanosoma brucei* (DeLange *et al.* 1983; Nelson *et al.* 1983); tubulin genes in *T. brucei* (Thomashow *et al.* 1983; Seebeck *et al.* 1983), *Leishmania enrietti* (Landfear *et al.* 1983), *L. major* (Spithill and Samarus, 1987) and *Leptomonos seymouri* (Bellofatto and Cross, 1988); hsp 70 genes in *Trypanosoma cruzi* (Requena *et al.* 1988); calmodulin genes in *T. brucei gambiense* (Tschudi *et al.* 1985); glyceraldehyde-phosphate dehydrogenase genes in *T. brucei* (Michels *et al.* 1986); ATPase in *Leishmania donovani* (Meade *et al.* 1987); phosphoglycerate kinase genes in *T. brucei* (Le Blancq *et al.* 1988); and the actin genes in *T. brucei* (Ben Amar *et al.* 1988). GP63, the major surface glycoprotein found in *Leishmania* has also been found to be expressed from multiple gene copies (Button *et al.* 1989; Webb *et al.* 1990; Miller *et al.* 1990; Steinkraus and Langer, 1992; Medina-Acosta *et al.* 1993).

Although no promoters have been identified in DNA of trypanosomatids, evidence suggests that tandemly repeated genes found in this family are expressed in a polycistronic fashion (Ben Amar *et al.* 1988; Button *et al.* 1989; Imboden *et al.* 1987; Muhich and Boothroyd, 1988). Transcription through the entire locus is thought to give rise to a precursor polycistronic mRNA that rapidly undergoes a process of *trans*-splicing and polyadenylation to give rise to the mature mRNA (for reviews see Perry and Agabian, 1991; Laird 1989; Agabian 1990). In the *trans*-splicing reaction, the mature transcript is formed via the splicing of two separately encoded gene

products. A 39 nucleotide splice leader sequence is derived from a 140 nucleotide transcript encoded by the mini-exon genes. The 39 nucleotide splice leader is spliced to the internal 3' acceptor site of the 5' terminus of the protein encoding precursor mRNA. Splicing of the two exons is thought to occur via the formation of a 2'-5' phosphodiester bond of a Y branched intermediate analogous to the lariat structure formed by introns in the *cis* splicing reactions of higher eukaryotes. Thus, as a result of processing via *trans*-splicing, all processed transcripts of trypanosomatids contain the 39 nucleotide spliced leader sequence at the 5' end. One possible function of *trans*-splicing is to provide 5' cap structures to the mature mRNA. The 5' end of the spliced leader like the 5' end of mRNAs in higher eukaryotes contains a m^7G cap which likely functions in mRNA stability and in the recognition of the transcript by the translational machinery (Freistadt *et al.* 1987; Perry *et al.* 1987). *Trans*-splicing may also be important for transport of the mature mRNA from the nucleus (Perry and Agabian, 1991). No introns have been identified in any genes of kinetoplastids studied to date and therefore the process of *cis* splicing has not been found to occur.

Another unusual feature found in trypanosomatids is the process of RNA editing. In this process, specific internal uridines are removed and/or added to the mRNAs transcribed in the mitochondrion (for review see Simpson, 1990; Perry and Agabian, 1991). The function of RNA editing is not known.

D. GP63: THE MAJOR SURFACE GLYCOPROTEIN OF LEISHMANIA

Molecules present on the surface of protozoan parasites play an important role in the defense strategy of these parasites in evading host immune defense mechanisms and are thus important in the virulence of these parasites. *Trypanosoma brucei*, the causative agent of African sleeping sickness undergoes a process of antigen variation whereby the variable surface glycoproteins (VSGs) are continually changed in order to avoid detection by the immune system of the host. The two most abundant molecules on the surface of *Leishmania* promastigotes are lipophosphoglycan (referred to as LPG) and GP63 and it has been demonstrated that they play important roles in the virulence of these disease causing parasites. The glycolipid LPG is the most abundant surface molecule on the surface of promastigotes with 2-5 million copies per cell (for a review of LPG see Turco, 1990). Evidence suggests that LPG undergoes developmentally regulated modifications during promastigote transition from a non-infectious stage found in the logarithmic phase of growth to the infectious or metacyclic stage found in stationary phase of growth. These changes were found to be correlated with resistance to blood complement factors (Sacks and daSilva, 1987). Furthermore, LPG was shown to inhibit hydrolytic enzymes and was therefore postulated to act as a barrier preventing hydrolases from reaching the parasite membrane. It was suggested that this barrier would offer the promastigote protection inside the digestive environment of macrophage phagolysosomes (El-On *et al.* 1980).

The most abundant protein present on the surface of promastigotes of all *Leishmania* species studied to date is GP63, a glycoprotein with a molecular weight of 63 kDa (Colmer-Gould *et al.* 1985; Bouvier *et al.* 1985; Etges *et al.* 1985; Chang and Chang, 1986; Bouvier *et al.* 1987; Frommel *et al.* 1989). GP63 is estimated to occur at approximately 500,000 copies per promastigote representing approximately 0.5 to 1.0% of promastigote protein (Bordier, 1987; Etges *et al.* 1986).

GP63 was found to be attached to the surface membrane of promastigotes by a glycosylphosphatidylinositol (GPI) anchor as is the membrane bound VSG of African trypanosomes (Bordier *et al.* 1986; Etges *et al.* 1986). Metabolic labeling of promastigotes with

[^{3}H] myristic acid, which is biosynthetically incorporated into GPI anchor moieties, followed by western blot analysis of GP63 showed that GP63 was labeled with [^{3}H] myristic acid. Treatment of purified [^{3}H] myristic acid labeled GP63 with phosphatidylinositol phospholipase C (PI-PLC), which releases GPI anchors from their associated protein, was shown to change GP63 from an amphiphilic form to a hydrophilic form. The change from the amphiphilic form to the hydrophilic form was accompanied by a loss of the incorporated label indicating loss of the GPI anchor with PI-PLC treatment.

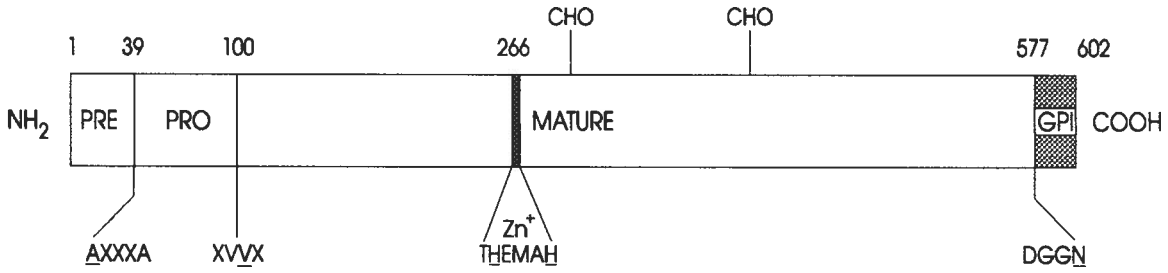
Characterization of GP63 demonstrated that GP63 is an *N*-linked glycosylated protein since treatment of the protein with deglycosylation enzymes or treatment of promastigotes with glycosylation inhibitors such as tunicamycin was shown to reduce the molecular weight of GP63 from 63 kDa to 54 kDa (Chang *et al.* 1986). Furthermore, GP63 was shown to possess protease activity (Etges *et al.* 1986). Enzymatic activity for *L. major* GP63 was determined to have neutral to alkaline pH optimum of pH 7-9 with activity being retained in detergent solubilized extracts or on the surface of living or fixed promastigotes (Etges *et al.* 1986). *L. mexicana amazonensis* GP63 on the other hand was shown to have an acidic pH optimum (Chaudhuri and Chang, 1988). Discrepancies in the observed pH optimum of GP63 from the two different species may have been due to differences in substrates used in enzyme assays (Tzinia and Soteriadou, 1991). GP63 was later demonstrated to be a zinc-binding metalloprotease requiring the presence of zinc for enzymatic activity (Bouvier *et al.* 1989; Jongeneel *et al.* 1989).

The first *Leishmania* gp63 DNA sequence was reported by Button and McMaster (1988, 1990) who cloned and sequenced a genomic DNA fragment that contained the sequence for *L. major* gp63. The single open reading frame of the *L. major* gp63 gene was predicted to encode a precursor protein of 602 amino acids containing four regions (Figure 3). The first region contained a hydrophilic prepeptide signal sequence (amino acids 1-39) likely responsible for translocation of the protein to the endoplasmic reticulum. The second region (amino acids 40-100) was predicted to form a propeptide regulatory sequence associated with regulation of the enzymatic activity of the protease (Button and McMaster, 1988; Bouvier *et al.* 1989; Macdonald *et al.* 1994). The third region (amino acids 101-577) was predicted to encode the mature form of

the protein and was shown to contain a zinc binding active site that was shown to be well conserved with other zinc metalloproteinases (Chaudhuri *et al.* 1989; Bouvier *et al.* 1989; Jongeneel *et al.* 1989). Two asparagines likely involved in *N*-linked glycosylation were also identified in the mature protein (Button and McMaster, 1988). The fourth region at the carboxy terminus was found to contain a hydrophobic sequence (amino acids 578-602) that is removed for addition of the glycophosphatidylinositol anchor for membrane attachment of the mature form of the protein (Button and McMaster, 1988; Schneider *et al.* 1990).

Figure 2. The predicted protein structure of GP63.

The main features of GP63 predicted from the DNA sequence of *L. major gp63* (Button and McMaster, 1988; Button and McMaster, 1990). **Pre**, prepeptide region; **Pro**, propeptide region; **Mature**, the mature protein following post translational processing; **GPI**, the carboxy terminus remove for attachment of the glycosylphosphatidyl inositol anchor; **CHO**, potential *N*-linked glycosylation sites for carbohydrate attachment to the mature protein. The zinc binding active site along with amino acid cleavage sites involved in processing are indicated. The position number of amino acids in each region is also shown. This diagram is adapted from Medina-Acosta *et al.* (1993).



Despite the wealth of information about GP63, the role of this enzyme in the biology of the parasite is still unknown. However, GP63 has been implicated in several important roles. Evidence suggests that GP63 is involved in the binding of the promastigotes to host macrophages with the binding being mediated by the macrophage complement receptor type three (CR3) molecule (CR3 has also been referred to as Mac-1, C3bi receptor, or CD11b/CD18) (Chang and Chang, 1986; Olafson *et al.* 1990; Russell and Wilhelm, 1986; Russell and Wright, 1988). Further analysis of promastigote-macrophage interactions indicated the requirement of opsonic complement to facilitate binding of promastigotes to CR3 receptors on macrophage cells. This requirement was demonstrated by Mosser *et al.* (1992) whereby the ability of *Leishmania* promastigotes to bind to purified CR3 or CR3 expressed on the surface of transfected COS cells was shown to be dependent on the presence of serum containing the complement factor C3. Parasites incubated in heat inactivated serum or serum which had been immunologically depleted of C3 did not bind to CR3 integrins. COS cells were used in this study because it was suggested that macrophage cells used in earlier GP63-macrophage binding assays may have secreted C3 to opsonize *Leishmania* cells resulting in phagocytosis. It had been previously been demonstrated that GP63 was an acceptor for the complement factor C3 (Russell, 1987). Taken together, these results suggested that one of the functions of GP63 is to bind and activate the complement component C3. Conversion of C3 to C3bi would result in opsonization of the parasite surface with C3bi enabling the parasite to bind the CR3 integrin on macrophage cells for phagocytosis. Conversion of C3 to C3bi would also inhibit further activation of the complement cascade that would be lethal to the parasites. Furthermore, attachment and uptake of promastigotes via the CR3 integrin would seem to be the ideal mode of entry for the parasite since phagocytosis via the CR3 receptor is not associated with the production of toxic superoxide radicals as are other receptors (Berton and Gordon, 1983; Wright and Silverstein, 1983).

The proteolytic activity of GP63 has been suggested to offer the parasite protection from degradation within the macrophage phagolysosome. Evidence for a protective role was provided by Chaudhuri *et al.* (1989) who demonstrated that proteins entrapped in liposomes coated with native GP63 were not degraded in the phagolysosome. It was hypothesized that the protease

activity of GP63 may inactivate or degrade lytic microbicidal factors in the phagolysosome of host macrophages.

The presence of GP63 in the promastigote stage of the parasites life cycle has been well documented which is in sharp contrast to reports for expression of the protease in the amastigote. Northern blot analysis indicated that gp63 transcripts were being synthesized in both life stages of *L. major* and *L. mexicana* (Button *et al.* 1989). Using anti-recombinant GP63 antibodies, Frommel *et al.* (1989) demonstrated the presence of GP63 in amastigotes of *L. mexicana* by western blot analysis and the presence of GP63 in *L. major* and *L. mexicana* amastigotes by immunofluorescence staining. Pimenta *et al.* (1991) using immunogold labeling detected GP63 on the surface of amastigotes of *L. major*. In contrast to these results, Schneider *et al.* (1992) failed to detect any significant level of GP63 in *L. major* amastigotes using polyclonal antiserum raised against deglycosylated GP63.

Further investigation of GP63 expression in the amastigote stage of *L. mexicana* suggested that most of the protein was localized to the flagellar pocket of amastigotes and that this form of GP63 lacked a GPI anchor (Medina-Acosta *et al.* 1989). Evidence for lack of a GPI anchor in amastigote GP63 came from the fact that the majority of GP63 immunoprecipitated from amastigotes was not metabolically labeled with [^{3}H] myristic acid. It was also estimated that *L. mexicana* amastigotes express GP63 at only 10% of levels found in promastigotes (Medina-Acosta *et al.* 1989). Recently, western blot analysis of water soluble proteins from *L. mexicana* indicated the presence of hydrophilic GP63-related proteins in *L. mexicana* amastigotes. Furthermore, using polyvalent antisera against native, hydrophilic GP63 in immunoelectron microscopy, a GP63-like protein in the amastigote stage was localized to the lumen of large lysosomes referred to as megasomes which are characteristic of this life stage of this species (Bahr *et al.* 1993).

E. GENES ENCODING GP63 IN LEISHMANIA

Genes encoding GP63 are found in multiple copy tandem arrays in all *Leishmania* species studied to date and are highly conserved. *L. major* has been shown to possess six copies of gp63 genes (Button *et al.* 1989) while *L. mexicana* contains ten gene copies (Medina-Acosta *et al.* 1993). At least seven GP63 gene copies are present in *L. chagasi* and more than ten copies are found in *L. donovani* (Miller *et al.* 1990; Button *et al.* 1989; Webb *et al.* 1991). Multiple copies of GP63 genes are also found on at least two separate chromosomes in *L. guyanensis* (Stienkraus and Langer, 1992).

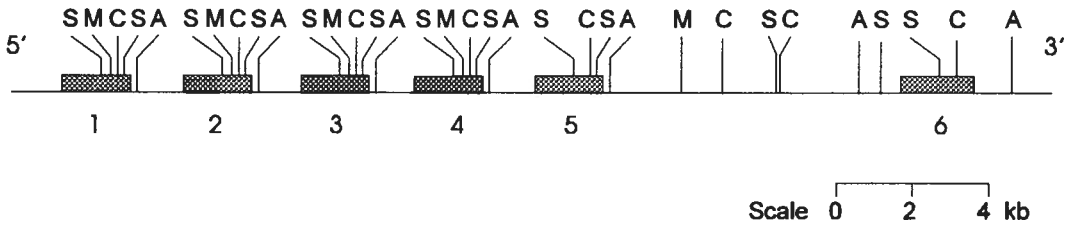
Recently, it has become clear that within a given species there are different forms of gp63 genes, some of which exhibit stage-specific expression in the parasite's life cycle. In *L. chagasi* promastigotes, there are three distinct classes of gp63 transcripts encoded by three different gene classes. Two of the different gp63 transcripts (referred to as *L1* and *S1*) were found to be differentially expressed in logarithmic phase (*L1*) and stationary phase (*S1*) of promastigote growth while the third class (referred to as *C1*) was found to be expressed during both log and stationary phases of the promastigote growth cycle (Ramamoorthy *et al.* 1992). Amastigote gp63 expression was not determined in this species. In *L. mexicana*, three gp63 gene classes were also found. Two of the gene classes referred to as *(C2)* and *(C3)*, were found to be expressed in the promastigote stage and were not detected in the amastigote stage in Northern blot analysis. The third gp63 class, referred to as *(C1)*, was shown to be expressed constitutively in both amastigotes and promastigotes. The *L. mexicana C1* gp63 encodes a predicted protein that has an extended carboxy terminus substantially different from the GPI anchor addition sequence found in other *Leishmania* species (Medina-Acosta *et al.* 1993). Recent evidence has suggested that this form of GP63 encoded by *(C1)* may be the GP63-like protein localized in the megasomes of *L. mexicana* amastigotes (Bahr *et al.* 1993). Interestingly, the *L. mexicana (C1)* gp63 gene shares a high degree of sequence identity to the *L. chagasi (C1)* gp63 gene.

F. FOCUS OF THIS STUDY

Previous analysis of the *L. major* gp63 locus has determined the organization of six gp63 genes (Button *et al.* 1989) as well as the sequence of the first gp63 gene in the locus (Button and McMaster, 1988; Button and McMaster, 1990). Five tandemly repeated genes were shown to be separated by a 1.3 kb intergenic region with a sixth gene separated from the five repeats by a distance of about 8 kb (see figure 3). Restriction map analysis of the gp63 locus indicated that the first five tandemly repeated genes were highly conserved. The sixth separated gene however was shown to be less conserved based on restriction map analysis and therefore indicated that this gene likely encoded a different form of GP63. Separation of the sixth gene from the five tandemly repeated genes also indicated the possibility of differential regulation and expression of *L. major* gp63 genes. Differential regulation of different forms of gp63 genes could be important in the expression of distinct forms of GP63 in the two life stages of the parasite. The objective of this thesis was to determine the structure and expression of the sixth *L. major* gp63 gene. In addition, the expression of the protein encoded by *L. major gp63-6* was investigated.

Figure 3. Physical map of the gp63 gene locus in *L. major*.

The six gp63 genes in *L. major* are found on a single locus of about 24 kb. The genes are numbered 1-6 in the 5' to 3' orientation. Restriction enzyme sites are indicated: A. *Sal* I; C, *Cla* I; M, *Sma* I; S, *Sst* I. This restriction map is a partial map adapted from Button *et al.* (1989). Note: the original restriction map (Button *et al.* 1989) had a *Sma* I site in gene 6 which upon subsequent restriction enzyme analysis was shown not to be present.



II. MATERIALS AND METHODS

A. LEISHMANIA STRAINS

1. Species and strains used.

The following WHO reference strains of *Leishmania* parasites were used: *L. major* WHOM/IR/-/173 (provided by Karen Dell, University of California San Francisco) and *L. donovani* (strain LV9) MHOM/ET/67/HU3.

2. Promastigotes.

Leishmania promastigotes were maintained in tissue culture medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 40 mM HEPES (pH 7.4), 50 units ml^{-1} penicillin, 50 $\mu g\ ml^{-1}$ streptomycin, 0.1 mM adenine, and 0.0005% Hemin. Cultures were maintained at densities ranging from 5×10^5 to 4×10^7 cells ml^{-1} and kept at 26°C in a non-humidified incubator.

3. Amastigotes.

To obtain *in vivo* amastigotes, 10^7 stationary phase ($> 2 \times 10^7$ cells ml^{-1}) promastigotes were injected into the base of the tail of BALB/c mice. Visible lesions developed in about four weeks and were allowed to expand for another four weeks. Animals were sacrificed and amastigotes were isolated by homogenizing lesion cells through a fine wire mesh screen into PBS.

B. VECTORS AND BACTERIAL STRAINS

The plasmid vector pBluescript KS+ (Stratagene, La Jolla, CA) was used for standard cloning procedures. Plasmids were maintained in DH5αF' *E. coli* cells (BRL, Gaithersburg, Maryland).

C. GENERAL MOLECULAR BIOLOGY TECHNIQUES

1. Agarose gel electrophoresis.

DNA and RNA were separated using 0.7%-1.2% agarose gels in 0.5X TBE electrophoresis buffer (44.5 mM Tris Borate and 1.25 mM EDTA, pH 8.3). DNA and RNA were visualized by incorporating 0.5 μg ml^{-1} ethidium bromide in gels or by staining gels in 0.5 μg ml^{-1} ethidium bromide.

2. Restriction enzyme analysis.

DNA for Southern blot analysis, restriction mapping or for cloning procedures was digested with 3-5 Units of restriction enzyme per μg of DNA. Reaction mixes in appropriate buffers supplied by restriction enzyme manufacturers were carried out at 37°C for at least one hour as described in Maniatis *et al.* (1989).

3. Gel purification.

DNA separated by agarose gel electrophoresis was purified using a Quiex agarose gel extraction kit (Quiagen Inc., Chatsworth, CA) following the instructions of the manufacturer.

4. Ligations and transformations.

Dephosphorylation of vector DNA 5' ends with calf intestinal alkaline phosphatase was carried out as described in Maniatis *et al.* (1989). Ligation reactions contained 50 - 100 ng of

vector DNA and 1-3X molar excess of insert DNA in ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$,10 mM DTT, 1 mM Spermidine HCl, 1 mM ATP pH 7.0, and 5 mg ml^{-1} BSA) with 1 - 10 units T4 DNA ligase in a total volume of 20 µl. Reactions were carried out at 15°C overnight.

Plasmid DNA was introduced into host *E. coli* cells made competent by the $CaCl_2$ method (Lederburg and Cohen, 1974). Approximately 10 - 40 ng of DNA was added to 200 µl of competent cells in 17x100 mm polypropylene tubes (Falcon 2059) on ice and allowed to incubate for 30 min. Cells were heat shocked at 42°C for 45 sec. Cells were then added to 0.9 ml of SOC media and incubated at 37°C for 1 hour with shaking. Cells were plated onto LB agar plates with appropriate antibiotic for selection of transformed cells and incubated overnight at 37°C. Colonies were picked and grown in LB media with appropriate antibiotic selection.

D. DNA ISOLATION

Large scale isolation of plasmid DNA used for sequencing, Southern blot analysis, and transfection experiments was carried out by the alkaline lysis method followed by CsCl density gradient ultracentrifigation (Maniatis *et al.* 1989). Small scale isolation of plasmid DNA for restriction enzyme analysis and sequencing was carried out by the "Speedprep" LiCl purification method as described in He and Kaderbhai, (1990).

E. RNA ISOLATION

Total promastigote, amastigote, or mouse spleen leukocyte RNA was isolated by the single-step acid guanidinium method (Chomczynski and Sacchi, 1987). Mouse leukocytes were obtained from the spleens of noninfected BALB/c mice by homogenizing spleen cells over a fine wire mesh screen. Cells were collected in PBS.

F. CLONING AND SEQUENCING OF L. MAJOR gp63-6

1. Cloning of *L. major gp63-6*.

A 4.1 kb *Sal* I fragment encompassing the entire coding region of *L. major gp63-6* along with approximately 1 kb of 5' and 1 kb of 3' flanking region from the *L. major* λEMBL3 phage clones λmms2-4 (described in Button *et al.* 1989) was subcloned into the *Sal* I site of the plasmid vector pBluescript SK+ to generate the clone pLGS6.1 (Linda Button, UBC, unpublished data).

2. DNA sequencing.

Double strand DNA sequencing was carried out using the dideoxy chain termination method (Sanger *et al.* 1977) using T7 DNA polymerase and 7-deaza-dGTP (Pharmacia Canada Ltd., Baie D'Urfe. Que.) or a Sequenase^{TM} system kit (US Biochemicals, Cleveland, OH) as indicated by the manufacturer except that 10% DMSO was added to the labeling reactions. Vector specific oligonucleotide primers (M13 forward, M13 reverse, T7, and T3) as well as *L. major gp63* specific oligonucleotide primers were used in sequencing. The sequencing strategy also employed the use of deletion mutant clones generated by sequential Exonuclease III digestion (Henikoff, 1984) using the Erase-a-base^{TM} system (Promega Corp., Madison, WI), as indicated by the manufacturer. Sequencing was carried out on both DNA strands.

2. Computer analysis of sequence data.

DNA and amino acid sequence data was analyzed using PC Gene 6.70 software (Intelligenetics Inc., Switzerland). Searches of GenBank and EMBL databank were conducted using the NCBI BLAST e-mail server (National Center for Biotechnology Information at the National Library of Medicine). Alignments of sequences used the method described in Altschul *et al.* (1990).

G. SOUTHERN BLOT CONDITIONS

Southern blot analysis was carried out as described in Maniatis *et al.* (1989). Briefly, restriction enzyme digested plasmid DNA was fractionated on 0.75% agarose gels at 100 V for 1 h. Gels were soaked in 0.25 M HCl and agitated gently for 15 min at room temperature to depurinate the DNA. DNA was then denatured by immersing gels in 1.5 M NaCl, 0.5 M NaOH and agitated gently for 30 min at room temperature. DNA was transferred to Hybond-N nylon filters (Amersham Corp., Oakville, Ont.) by vacuum blotting in 10X SSC (1.5 M NaCl, 0.15 M Na citrate pH 8.3) using a Bio-Rad Model 785 Vacuum Blotter (Bio-Rad, Richmond, CA) as indicated by the manufacturer. DNA was crosslinked to filters by exposure to UV light from a transilluminator for 2-5 min. Prehybridization and hybridization conditions were carried out at 65 °C in 6X SSC, 5X Denhardt's mix (0.1% bovine serum albumin (BSA), 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, and 50 μg ml^{-1} denatured carrier DNA. Filters were washed at room temperature for 10 minutes in 2X SSC, 0.1% SDS, and at 65°C for two 30 minute washes in 2X SSC, 0.1% SDS, followed by two fifteen minute high stringency washes at 65°C in 0.1X SSC, 0.1% SDS. Filters were rinsed with 0.1X SSC at room temperature and exposed to XAR-5 film (Kodak) with an intensifying screen at -70°C.

H. NORTHERN BLOT CONDITIONS

Northern hybridization analysis was carried out as described (Maniatis *et al.* 1989). RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde with electrophoresis in MOPS buffer (20 mM morpholinopropane sulfonic acid, 5 mM sodium acetate, 0.1 mM Na_2EDTA, pH 7.0) at 50 V for 3h. RNA was transferred to Hybond-N nylon filters by capillary blotting in 20X SSC (3 M NaCl, 0.3 M Na Citrate pH 8.3). RNA was covalently attached to the filter by exposing filters to UV light from a transilluminator for 2-5 min. Prehybridization and hybridization conditions as well as wash conditions were carried out as described under Southern

blot conditions. Blots used for rehybridization were stripped by washing the membrane at 65°C in 5 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1X Denhardt's mix for 2 hours. Autoradiography was carried out for the maximum exposure time of subsequent hybridization exposure times to confirm removal of probes.

I. HYBRIDIZATION PROBES

Polymerase chain reaction (PCR) (Saiki *et al.* 1988) was used to generate hybridization probes specific to *L. major gp63-1* and *L. major gp63-6*. Oligonucleotides used to generate a 232 bp PCR product specific to *L. major gp63-1* were: 5'AACACGGCGGCTGGTCGTCG3' (position 1729 to 1748; Fig. 3, *Lm gp63-1*) and 5'CTTCCGAAAGACAATGAGAGGG3' (complementary to position 1940 to 1961; Fig. 3, *Lm gp63-1*). Oligonucleotides used to generate a 260 bp PCR product specific to *L. major gp63-6* were: 5'GGAGTAGTTCTCTCTCTCATGG3' (position 1840 to 1861; Fig. 3, *Lm. gp63-6*) and 5'CACCGCAGAAGACGAGGGACGG3' (complementary to position 2079 to 2100; Fig. 3, *Lm gp63-6*). The plasmid pBS10Rb.1 (Button and McMaster, 1988) containing the *L. major gp63-1* and pLGS6.1 containing *L. major gp63-6* were used as gene 1 and gene 6 templates respectively. 100 ng of plasmid template DNA was amplified using a Perkin Elmer Cetus 9600 thermal cycler. The PCR conditions were 96°C, 1 min., 62°C, 1 min., and 72°C, 1 min. for 30 cycles using AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, New Jersey). All reaction mixes contained 10% DMSO. Gel purified PCR products were labeled to high specific activity (> 10^8 cpm ug^{-1}) by the random priming method (Feinberg and Vogelstein, 1983) in the presence of [α^{33}P] dATP or [α^{32}P] dCTP.

A 0.85 kb *Eco* RI fragment of the *L. major* β-tubulin cDNA clone pP121/4 (Spithill and Samarus, 1985) was labeled as described above and used in Northern blot analysis.

J TRANSFECTION OF L. DONOVANI AND COS-7 CELLS WITH L. MAJOR gp63-1 AND L. MAJOR gp63-6

1 PCR amplification of *L. major gp63-6.*

PCR was used to generate a *L. major gp63-6* product with 5' and 3' *Bam* HI cloning sites flanking the coding sequence. PCR primers used were: 5'CCTGCGGATCCATGTCCGTGGACA3' (position -11 to 13; Fig. 3); and 5'GGCCGCGGATCCTCACGTCGGGAGCCCC3' (complementary to position 1920 to 1947; Fig. 3, *Lm gp63-6*). (The *Bam* HI site is underlined and the initiation and stop codons are shown in bold type). Reaction mixes contained 5 ng of pLGS6.1 template DNA and used *Ultma*^{TM} DNA polymerase (Perkin Elmer) as instructed by the manufacture with the addition of 10 % DMSO in reaction mixes. *Ultma*^{TM} DNA polymerase was used since it contains the 3' to 5' exonuclease proofreading function and therefore reduces the possibility of misinsertion and misextension in PCR. Cycling conditions were as follows: 94°C, 1 min, 55°C, 2 min, and 72°C, 5 min with a 10 second extension per cycle for a total of 30 cycles.

2. Cloning of *L. major gp63-1* and *L. major gp63-6* into the *Leishmania* expression vector pLEX.

The *Leishmania* expression vector pLEX has previously been described (Webb, 1993). A *L. major gp63-1* PCR product kindly provided by Charlotte Morrison and described in (Macdonald *et al.* 1994) and the *L. major gp63-6* PCR product described above were individually subcloned into the pLEX vector as follows. The gene 1 and 6 PCR products were digested with *Bam* HI. The 5' overhang ends were blunt ended by filling in with Klenow and dNTPs as described in (Maniatis *et al.* 1989). The blunt ended PCR products were individually cloned into the *Sma* I site of the multiple cloning site of the pLEX vector to generate the clones pLEXLMGP63-1 (containing gene 1) and pLEXLMGP63-6 (containing gene 6).

3. Cloning of *L. major gp63-1* and *L. major gp63-6* into the COS cell expression vector PAX 111.

The COS cell expression vector PAX 111 has been previously described (Kay *et al.* 1990; Kay and Humphries, 1991). *L. major gp63-1* was previously cloned into the *Bam* HI site of PAX 111 (Macdonald *et al.* 1994) and was kindly provided by Mary Macdonald. The *L. major gp63-6* PCR product described above was digested with *Bam* HI and cloned into the *Bam* HI site of the multiple cloning site of the PAX 111 expression vector. The two clones are referred to as PAXLMGP63-1 and PAXLMGP63-6 respectively. Plasmids were maintained DH5αF'::p3 *E. coli* cells.

4. Transfection of *Leishmania.*

L. donovani promastigotes were transfected with the plasmid DNA by electroporation as previously described (Kapler *et al.* 1990). Briefly, *L. donovani* promastigotes in mid-logarithmic growth phase (5×10^6 to 1×10^7 cells ml^{-1}) were collected by centrifugation, washed once in ice cold electroporation buffer (21 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and resuspended at 1×10^8 cells ml^{-1} in electroporation buffer. Promastigotes (0.4 ml) were mixed with 20 μg of sterile DNA in 0.2 cm gap width electroporation cuvettes and electroporated using a Gene Pulser Apparatus according to the manufacturer's instructions (BIO-RAD). The apparatus was set at a voltage of 0.45 kV and a capacitance of 500 μFd. Electroporated cells were immediately placed on ice for 10 min before being moved to 25 ml tissue culture flasks containing 10 ml of drug free media. Cells were incubated for 24 hours to allow for expression of the transfected genes. Transfectants were collected by centrifugation and plated onto semi-solid M199 plates containing 1% agar and 32 μg ml^{-1} hygromycin B (Sigma, St. Louis, MO) for selection. Clonal selection of transfectants was carried out by picking colonies arising on drug selection plates and transferring them into 1 ml of M199 media containing 16 μg ml^{-1} hygromycin B and expanded in the presence of the selective drug.

5. Transfection of COS-7 cells.

COS-7 cells (Cell 23,174-182 American Type Culture Collection, catalogue number CRL 1651, Rockville, MD.) were transfected with plasmid DNA by electroporation as previously described (Kettleborough *et al.* 1992). COS cells were grown to subconfluence in DMEM media (Gibco Laboratories) supplemented with 10% FCS. Cells were washed with PBS and briefly treated with 1 ml 0.05% trypsin (Gibco Laboratories) to lift cells off of tissue culture flasks. The trypsin was inactivated by transferring the cells into 1 ml of FCS. Cells were washed once with PBS, centrifuged, and resuspended in PBS at 1 x 10^7 cells ml^{-1}. The cells (0.8 ml) were mixed with 10 μg of sterile DNA in 0.4 cm wide gap cuvettes and electroporated with the Gene Pulsar apparatus set at a voltage of 1.9 kV and a capacitance of 25 μFd. Cells were incubated at room temperature for 10 minutes before being transferred to 14 ml of DMEM media in each of two tissue culture plates (100 cm^2) and incubated at 37°C for 72 hours. Cells were washed twice with PBS and harvested with 26 mM EDTA in PBS at room temperature and transferred to 1 ml of FCS. Cells were washed twice in PBS before preparation of lysates.

K. ANTIBODIES

Mouse monoclonal antibodies CP3.235 and CP3.139 were raised against the mature protein portion of recombinant GP63 (Button *et al.* 1991) and were used in western blot analysis at the indicated dilution. The monoclonal antibody #96 (Macdonald *et al.* 1994) was used in fluorescence flow cytometry analysis to study surface expression of GP63.

L. FLUORESCENCE FLOW CYTOMETRY ANALYSIS

Leishmania promastigotes were prepared for fluorescence flow cytometry analysis as follows. All steps were performed on ice. Promastigotes (5 x 10^6 cells) were washed in PBS containing 0.5% BSA and collected by centrifugation. Promastigotes were incubated on ice for 1 hour in 100 µl mAb #96 supernatant (or 0.5% BSA/PBS for control cells). The cells were washed twice in 0.5% BSA/PBS and resuspended in 50 µl of fluorescein-conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc.) diluted to 10 µg ml^{-1} in 0.5% BSA/PBS. Promastigotes were washed again in 0.5% BSA/PBS and resuspended in 0.4% formaldehyde in PBS and incubated on ice for 15 min to fix the cells. The promastigotes were washed in 0.5% BSA/PBS and resuspended in 0.5 ml 0.5% BSA/PBS and subjected to analysis by flow cytometry on a FACScan analyzer (Becton Dickinson Immunocytometry systems, San Jose, CA). Data was analyzed using Lysis II software (Becton Dickinson).

M. WESTERN BLOT ANALYSIS

Cells used for lysates were washed twice in ice cold PBS. Lysates were prepared by resuspending the cells in 0.2% Zwittergen in TBS, pH 8.5 at 1 x 10^5 to 1 x 10^6 cells ml^{-1}. Lysates were incubated on ice for 10 min and nuclei were pelleted by a 5 min spin at 13,000 x g.

Cell lysates (1 x 10^5 to 1.5 x 10^6 cells) were treated with or without 50 mU Endoglycosidase F (Boehringer Mannheim, Laval, Quebec) at 37°C overnight in the presence of Endoglycosidase F buffer (50 mM KH_2PO_4 pH 7.5, 1% SDS, 1 % Triton X-100, 20 mM EDTA and 1 % β-mercaptoethanol). Samples were denatured by boiling lysates in Laemmli sample buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl pH 6.8, 0.2% Bromophenol blue) containing 65 mM DTT prior to fractionation on a 8% separating gel by SDS-Polyacrylamide gel electrophoresis using a mini PROTEAN II system (Bio-Rad). Gels was immersed in semi-dry transfer buffer (48 mM Tris, 39 mM Glycine, 20% methanol, and 0.0375% SDS) for 15 min.

Separated proteins were transferred to immobilon membrane (Millipore, Mississauga, Ont.) for 45 min at 15 V using a Bio-Rad Trans-Blot SD® Semi-dry Transfer Cell as instructed by the manufacturer. Blots were incubated in blocking buffer (0.5% Tween 20, 0.5% BSA) at 37°C for 1 hour to overnight. Membranes were then incubated with indicated antibodies diluted in 0.5% BSA, 0.1% Tween 20 for 1 hour. After three washes (0.1% BSA, 0.5% Tween 20), membranes were incubated for 1 hour with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Bio-Rad) diluted 1:3000 in 0.5% BSA, 0.1% Tween 20. Blots were washed once in TBS followed by two washes in alkaline phosphatase buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$) and developed with BCIP/NBT solution as indicated by the manufacturer (BRL).

III. RESULTS

A. DNA AND PREDICTED AMINO ACID SEQUENCE OF L. MAJOR GP63-6

Previous analysis of *L. major* DNA determined GP63 to be encoded by six genes at a single chromosomal locus. Five of the genes were shown to be tandemly repeated with a sixth gene separated from the tandem array by approximately 8 kb (Button *et al.* 1989). Restriction map analysis suggested that the sixth gene (*L. major gp63-6*) was less conserved than the other five genes. To investigate a different form of GP63 in *Leishmania*, the complete DNA sequence of *L. major gp63-6* was determined and compared to the sequence of *L. major gp63-1* (Button and McMaster, 1988; Button and McMaster, 1990).

Previous mapping analysis of the *L. major* gp63 locus determined that the *L. major* λ EMBL3 phage clone λmms2-4 contained a 4.1 kb *Sal* I fragment which encompassed *L. major gp63-6* (Button *et al.* 1989). This 4.1 kb *Sal* I fragment containing the entire coding region of *L. major gp63-6* along with 1.1 kb of 5' and 1 kb of 3' flanking regions was subcloned into the *Sal* I site of the plasmid vector pBluescript KS+ to generate the clone pLGS6.1 for subsequent sequencing analysis (Linda Button, UBC, unpublished data).

In the present study, the complete DNA sequence of *L. major gp63-6* along with 0.3 kb of 5' and 1 kb of 3' flanking regions was determined by double strand sequencing of pLGS6.1 using the Sanger dideoxy chain termination method (Sanger *et al.* 1977). The complete nucleotide sequence of *L. major gp63-6* and flanking regions is shown in Figure 4 and is compared to the sequence of *L. major gp63-1*. Position 1 of the sequences corresponds to the ATG translational initiation codon. A three nucleotide gap was introduced in the *L. major gp63-1* sequence at position 1306-1308 for optimal alignment of the two sequences. Analysis of *L. major gp63-6* DNA sequence indicated an open reading frame of 1932 base pairs. In comparison, the open reading frame of *L. major gp63-1* was only 1806 base pairs long. The open reading frames of the

two *L. major* gp63 genes were found to share 94.9% identity at the DNA level indicating a high degree of conservation.

Comparison of the 5' flanking sequence indicated that the 193 nucleotides immediately 5' to the translation initiation codon (positions -1 to -193) were identical for the two *L. major* gp63 sequences. Beyond the 193 identical base pairs 5' to the initiation codon, the sequences were found to diverge. The sequence at positions -129 to -150 is consistent with the consensus 3' splice site signal sequence $[C/U]_nNNAG$ found 5' of all trypanosomatid protozoan protein encoding genes examined to date (Borst, 1986). The splice site signal sequence is proposed to be important in the *trans*-splicing reaction involved in processing of mRNA whereby the 39 nucleotide spliced leader transcript (encoded elsewhere in the genome) is attached to the nucleotide immediately 3' of the splice leader signal sequence found in the 5' untranslated region of the unprocessed protein encoding transcript (Borst, 1986; Goldburg *et al.* 1986).

Figure 4. The DNA sequence of *L. major gp63-6* and *L. major gp63-1*.

The nucleotide sequence for *L. major gp63-6* (*Lm gp63-6*) and *L. major gp63-1* (*Lm gp63-1*, Button and McMaster, 1988; Button and McMaster, 1990) is displayed in the 5' to 3' direction with the first base of the ATG initiation codon of the two genes labeled at position 1. The **ATG** initiation codon and termination codons **TAG** and **TGA** are indicated in bold type. Dots indicate nucleotides in *L. major gp63-1* which are identical to nucleotides in *L. major gp63-6*. Dashes indicate gaps introduced for optimal alignment of the sequences. A line is shown above the proposed splice leader acceptor site signal sequence at positions -129 to-150 in the 5' flanking region. Underlined sequences represent primer binding sites used in PCR to generate hybridization probes specific to *L. major gp63-6* and *L. major gp63-1*. Positions of base pairs are indicated.

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Lm gp63-6  gagtggcggggtgctgtccccctcgctgcggcgtccctccccgcgccagcacgcacgcactcccgtgcccgtgacccccctccgcaccagcaccgatgcg -201
Lm gp63-1  tct.c.gtttc.cgca.ggaaaa.aaagcgtt.cc.tga.ttaaacatct.c..ag...ca.t.taca..acctctatgacaac.tga.tg.g.acactc -201

Lm gp63-6  cactccgctctccctcccctcgcaccctccctcgcccatcccttgccgcctccctgtcccctccctccccagatccgccaacgcatccgatcccgctaca -101
Lm gp63-1  .gaattc..................................................................................... -101

Lm gp63-6  ccctctcccccgcccatacgcacgcgcacaccgccgtgcacaagccctcgccctcgccaccacacccccactgcccacagcgcccccgcgcctgcagagcc -1
Lm gp63-1  ..................................................................................... -1

Lm gp63-6  ATGTCCGTGGACAGCAGCAGCACGCACCGGCGCCGCTGCGTCGCCGCGCGCCTGGTGCGCCTCGCGGCTGCCGGCGCCGCAGTCACCGTTGCTGTCGGCA  100
Lm gp63-1  .....  100

Lm gp63-6  CCGCGGCCGCGTGGGCACACGCCGGTGCGCTGCAGCACCGCTGCGTCCACGACGCGATGCAGGCACGCGTGCGGCAGTCGGTGGCGGACCACCACAAGGC  200
Lm gp63-1  .....  200

Lm gp63-6  CCCCGGCGCGGTGTCCGCGGTGGGTCTGCCGTACGTTACTCTCGACGCCGCGCACACCGCGGCCGCCGCCGATCCCAGGCCGGGCAGCGCGCGCAGCGTC  300
Lm gp63-1  .....  300

Lm gp63-6  GTGCGCGACGTGAACTGGGGCGCGCTGCGCATCGCCGTCTCCACCGAGGACCTCACCGACCCCGCCTACCACTGCGCTCGCGTCGGGCAGCGTGTCAACA  400
Lm gp63-1  .....A......AG  400

Lm gp63-6  ACCACGCCGGCGCCATCGTCACCTGCACCGCCGAGGACATCCTCACCAACGAGAAGCGCGACATCCTGGTCAAGCACCTCATCCCGCAGGCGGTGCAGCT  500
Lm gp63-1  .....  500

Lm gp63-6  GCACAAGGAGCGGCTGAAGGTGCAGCAGGTGCAGGGCAAGTGGAAGGTGACGGACATGGTCGGCGAGATCTGTGGCGACTTCAAGGTGCCGCAGGCGCAC  600
Lm gp63-1  .....C.....T.....  600

Lm gp63-6  ATCACCGAGGGCTTCAGCAACACCGACTTCGTGATGTACGTCGCCTCCGTGCCGAGTGAGGAGGGTGTGCTGGCGTGGGCCACGACCTGCCAGACGTTCT  700
Lm gp63-1  .....  700

Lm gp63-6  CTGACGGCCATCCAGCCGTGGGCGTCATCAACATCCCCGCGGCGAACATTGCGTCGCGGTACGACCAGCTCGTCACGCGTGTCGTCACGCACGAGATGGC  800
Lm gp63-1  .....  800

Lm gp63-6  GCACGCGCTCGGCTTCAGCGGCCCATTCTTCGAGGACGCCCGCATCGTGGCGAACGTTTCGAACGTTCGAGGCAAGAACTTCGATGTTCCCGTGATCAAC  900
Lm gp63-1  .....C.....  900

Lm gp63-6  AGCAGCACGGCAGTGGCGAAGGCGCGCGAGCAGTACGGCTGCGACACCTTGGAGTATCTGGAGGTGGAGGACCAGGGCGGTGCGGGCTCCGCCGGGTCGC  1000
Lm gp63-1  .....  1000

Lm gp63-6  ACATCAAGATGCGCAACGCGCAGGACGAGCTCATGGCGCCTGCTGCAGCTGCCGGGTACTACACCGCCCTGACCATGGCCATCCTCCAGGACCTCGGCTT  1100
Lm gp63-1  .....T.....  1100

Lm gp63-6  CTACCAGGCGGACTTCAGCAAGGCCGAGGTGATGCCGTGGGGCCAGAACGCCGGCTGCGCCTTCCTCACCAACAAGTGCATGGAGCAGAGCGTCACGCAG  1200
Lm gp63-1  .....  1200

Lm gp63-6  TGGCCGGCGATGTTCTGCAATGAGAGCGAGGACGCCATCCGCTGCCCCACCAGTCGTCTCCTCCTCGGAACGTGTGGTATAAGGGAATACGAACCGCCGT  1300
Lm gp63-1  .....AG......TG.A..C...G.T.CCCGTC..CCGGGC.TTC  1300

Lm gp63-6  TGCCGCGATACTGGCAGTACTTCACCAACGCGTCTCTTGGGGGCTACTCGCCATTCCTGGACTACTGCCCGTTTGTTATCGGCTACGCTGATGGCTCGTG  1400
Lm gp63-1  C....---.....GG..C....C..C.CC...GT...CG.C...A.....TG.C..GG.GCC....AG.....AGC..  1397

Lm gp63-6  CAATCAGGACGCATCGTCGGCAGAGGAGTTCTTCACTGCATTCAACGTCTTCTCTGACGCGGCGCGCTGCATCGATGGCGCCTTCAGACCGAAGGCAACT  1500
Lm gp63-1  ..CG...CGT..C..TGA....C.T.CT.CG..GCTGC.C.....T.....  1497
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Lm gp63-6  AACGGCATAGTCAAGTCGTACGCCGGCCTGTGCGCCAACGTGCAGTGTGACACGGCCACACGCACGTACAGCGTGCAGGTGCACGGCAGTAACGACTACA 1600
Lm gp63-1  G.............................................................................................. 1597

Lm gp63-6  CCAACTGCACGCCGGGCCTCAGAGTTGAGCTGAGCACCGTGAGCAAAACCTTCGAGGAAGGCGGCTACATCACGTGCCCGCCGTACGTGGAGGTGTGCCA 1700
Lm gp63-1  ..........................................CG.........GG..................................... 1697

Lm gp63-6  GGGCAACGTGCAGGCTGCCAAGGACTTTGATGGCGACTCCGACAGCTCCAGCAGCTCCAGTGACGCTGCCGACAAGGCGGCGATTGAGCGGTGGAATGAG 1800
Lm gp63-1  ......................GGC.GCAA.ACGG.G.CTG.TCGTC.TG.TC.GC.C.C...G..GACGGC.CT.CT.G.G.CCGC.CT.CTG.CC 1797

Lm gp63-6  AGGATGGCCGGCCTGGCTACTGCGGCGACGGTGCTGCTAGGAGTAGTTCTCTCTCTCATGGCACTCGTCGTGGTGTGGCTACTGCTTGTCAGCTGCCCCA 1900
Lm gp63-1  GT.GC.CT.TAGgca.tggacag.a..ggt.ccggtggc.tgtcccc.gctc.c.ctccctgt.g.cggcg.agca...gcac..c.cc.gt.ca..a.. 1897

Lm gp63-6  GGTGGTGTTGCAAAGTTGGGGGGCTCCCGACGTGAgggacggcggcctgcatgcttgaaaaggagtgaaaaggatggctgtcggcgagatggcagcgaca 2000
Lm gp63-1  cacacacaca..c.ca..tacac.ct..ctttccccctc.ct.cct..cat..tc.ttcggaagag.g..tt                          1969

Lm gp63-6  aagtgtagtggcagcctgtccccatgccggcggccaagggtgcgcacgtgcgcggagtgctattattattcttattcgccgtccctcgtcttctgcggtg 2100

Lm gp63-6  caccatcggagtctgagctgggcgtgtgtttgggtgagtggggccgtttcacagcctccgcctccgtcccttccctcctactgctgtgggtgtgtgcgtg 2200

Lm gp63-6  ccgccgacaagcaccttaagctcttggtccttcaccgcgcagtggcacagatgattatcaaggtcagcgggcggctctcgacttatctcgcggtgccgtc 2300

Lm gp63-6  ttgaccacaatgtgtgcctcagtttttttttttgtggcgtttttctgctttgagctgttctgcggttttgccgcactggatagggcgtgcggcagacgta 2400

Lm gp63-6  ggcgggctagtgtgcgccctccctccctctctctctctgccgtaccccccccccctcctcctccacccatccctgctgcttcttctttgttttttgccttt 2500

Lm gp63-6  ccttgactgcacggcgcgcagaagcatcaaaacccctttcctccgcactgcctggacgctgcgtgccatcataataggtgtctctgtcagcgctcaggcga 2600

Lm gp63-6  aaggcatatggcttcgtctactttggtaatgagcttctgttgctgttttccgcaccgatgctgatcccctctttacgttgcgtcactcatctcctcgccc 2700

Lm gp63-6  ccctccccttctgtcccgcttgtgatgccatgccgccagtcggaggaggggaaggggggagcgtaggagagggtgagtcgcttgcttgtcgtacggtgttc 2800

Lm gp63-6  ttctttctcccttgctcgtggtgcggcggttgtgtagttcgcccgacaatgatgcgcgcacgtcatcgtgcatcaacgctcagggaatggcatcacacaa 2900

Lm gp63-6  cggagcgaaaaggcagctgagctgtcgac                                                                      2929
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A DNA alignment of the 5' flanking regions of all gp63 DNA sequences found in the data base was carried out to compare the 5' regions of the two *L. major* gp63 genes with gp63 genes found in other *Leishmania* species. Results of the alignment are shown in Figure 5. With the exception of the *L. guyanensis* sequences, all other gp63 5' flanking regions share a high degree of similarity within the 193 bp region 5' of the coding sequence. The proposed splice leader signal sequence (position -129 to -150) in both *L. major* gp63 sequences is identical to those found in the *L. chagasi*, *L. donovani* and *L. mexicana (C1)* gp63 sequences. No eukaryote transcription promoter elements were found in the 5' flanking region of *L. major gp63-6* or in any of the other *Leishmania* gp63 5' flanking sequences.

The 3' flanking region of *L. major gp63-6* was compared to the 3' regions of other gp63 genes. Alignment of gp63 3' flanking regions which were found to be similar to the 3' flanking sequence of *L. major gp63-6* is shown in Figure 6. The 3' flanking region of *L. major gp63-6* was shown to share considerable identity with the 3' flanking sequence of the *L. mexicana (C1)* gp63 and *L. chagasi (C1)* gp63. In contrast, the 3' flanking region of *L. major gp63-6* shared little identity with the 3' region of *L. major gp63-1* (see Fig. 4).

Figure 5. Comparison of *Leishmania* gp63 5' flanking sequences.

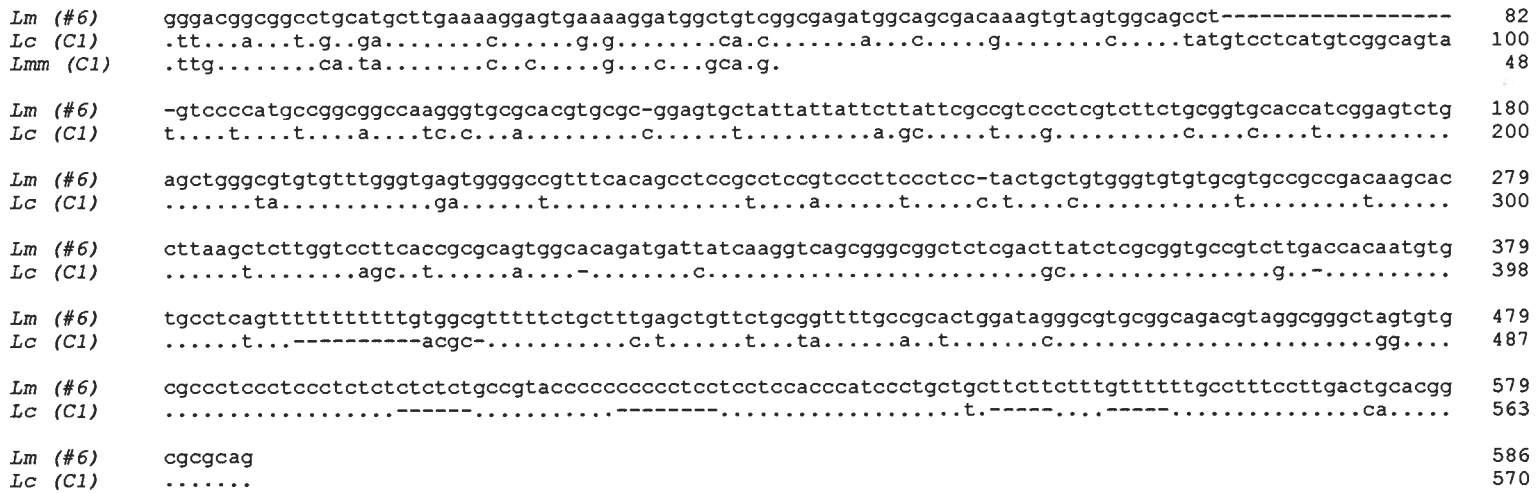
Lm (#6), *L. major gp63-6*; ***Lm (#1)***, *L. major gp63-1* (Button and McMaster, 1988; Button and McMaster, 1990); ***Lc***, *L. chagasi* gp63 (Miller *et al.* 1990); ***Ld***, *L. donovani* gp63 (Webb *et al.* 1991); ***Lmm (C1)***, *L. mexicana (C1)* gp63 (Medina-Acosta *et al.* 1993); ***Lc (S1)***, *L. chagasi (S1)* gp63 (Ramamoorthy *et al.* 1992); ***Lc (C1)***, *L. chagasi (C1)* gp63 (Ramamoorthy *et al.* 1992); ***Lc (L1)***, *L. chagasi (L1)* gp63 (Ramamoorthy *et al.* 1992); ***Lg (N)***, *L. guyanensis* (Steinkraus and Langer, 1992); ***Lg (K,X,Y, and Z)***, *L. guyanensis* cDNA clones (Steinkraus and Langer, unpublished data). Sequences are presented in the 5' to 3' direction with -1 being the nucleotide immediately upstream of the ATG initiation codon. Dots represent identical nucleotides and dashes represent gaps introduced for optimal alignment. A line is shown above the proposed splice leader acceptor site signal sequence at positions -129 to -150 in *Lm (#6)*.

Figure 5. Comparison of *Leishmania* gp63 5' flanking sequences.

Figure 6. Alignment of *Leishmania* gp63 3' flanking sequences.

Sequence alignment of *Leishmania* gp63 3' flanking regions that are similar to the *L. major gp63-6* 3' flanking region is shown in the 5' to 3' direction. ***Lm (#6)***, *L. major gp63-6*; ***Lc (C1)***, *L. chagasi (C1)* gp63 (Ramamoorthy *et al.* 1992); ***Lmm (C1)***, *L. mexicana (C1)* gp63 (Medina-Acosta *et al.* 1993). Position 1 represents the first nucleotide following the stop codon. Dots represent identical nucleotides and dashes represent gaps introduced for optimal alignment.

Figure 6. Alignment of *Leishmania* gp63 3' flanking sequences.

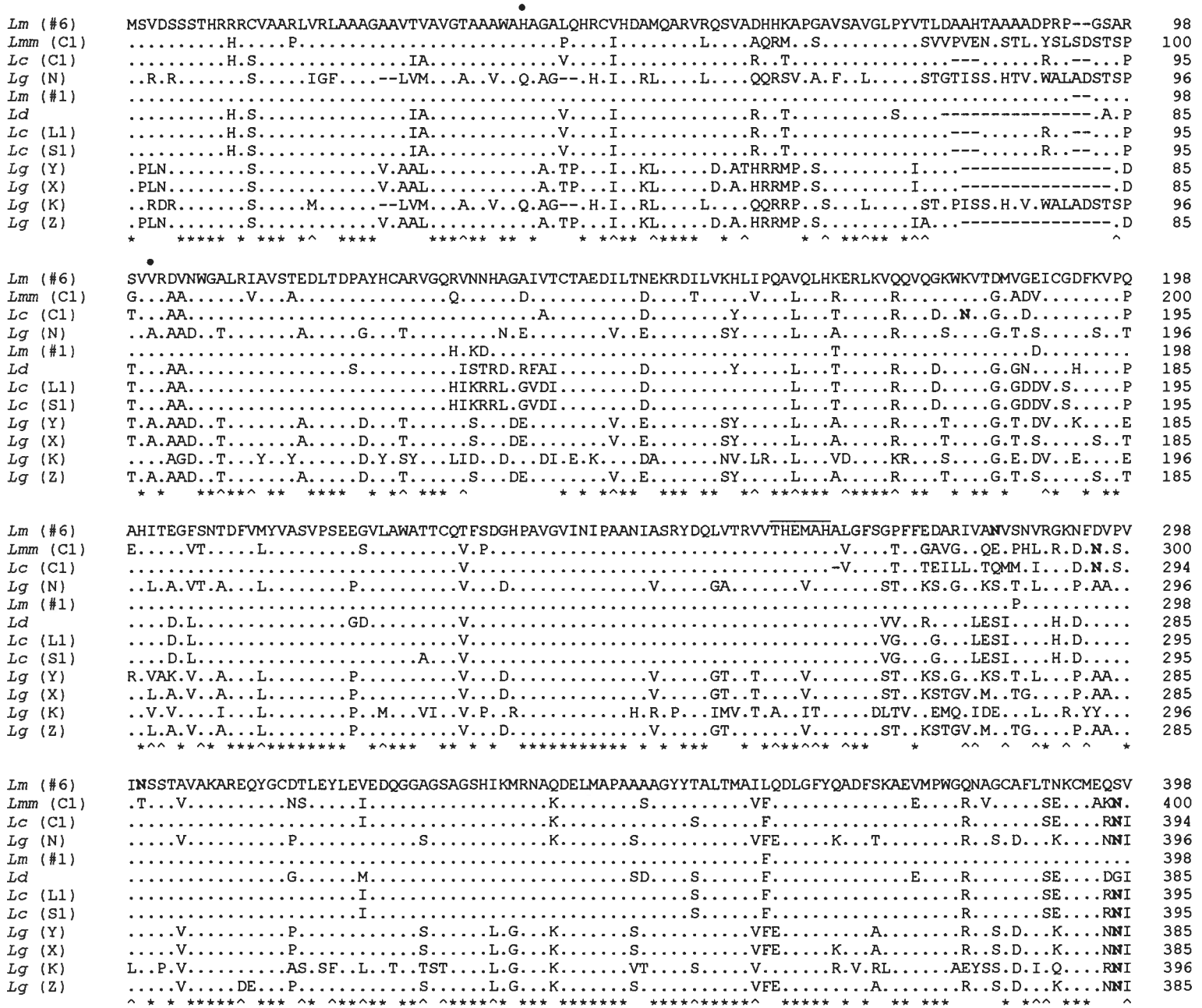


Comparison of the predicted proteins encoded by *L. major gp63-6* and *L. major gp63-1* as well as those encoded by gp63 genes of other *Leishmania* species is shown in Figure 7. Figure 8 depicts the amino acid sequences of *L. major* GP63-6 and *L. major* GP63-1 and highlights the main features and regions of differences of the predicted proteins. At the amino acid level, the two predicted *L. major* GP63 proteins share 90.4% identity. The open reading frame of *L. major gp63-6* however encodes a protein predicted to be 42 amino acids longer than the protein encoded by *L. major gp63-1*. Due to the high degree of similarity between the two forms of *L. major* GP63, *L. major* GP63-6 is predicted to be synthesized as a precursor protein which undergoes post translational modifications similar to *L. major* GP63-1 (Button and McMaster, 1988; Button and McMaster, 1990). The first 39 amino acids that encode the prepeptide signal sequence thought to be involved in translocation of the protein to the endoplasmic reticulum (Button and McMaster, 1988; Bouvier *et al.* 1989) were found to be identical to the sequence of the *L. major* GP63-1 (Fig. 7 and 8). The 61 amino acids which form the propeptide regulatory region (amino acids 40-100) involved in regulation of the protease activity of the protein (Button and McMaster, 1988; Bouvier *et al.* 1989; Bouvier *et al.* 1990; Macdonald *et al.* 1994) were also shown to be identical to the *L. major* GP63-1. A single cysteine at position 48 in the propeptide region was conserved between the two sequences. This single cysteine has been proposed to play a role in the cysteine switch mechanism whereby this cysteine is thought to interact with a zinc ion in the active site of the mature protein maintaining the latency of the proenzyme. Disruption of the interaction of the cysteine with the zinc ion is thought to result in activation of the protein (Springman *et al.* 1990; Bouvier *et al.* 1990; Goldberg *et al.* 1986; VanWart and Birkedal-Hansen, 1990). Comparison of the *L. major* sequences with those of other *Leishmania* species indicates that the pre-propeptide region is well conserved among all GP63 proteins (see Fig. 7).

The *L. major* GP63-6 protein was found to contain a putative zinc binding active site sequence (position 263-268, Fig. 7). This sequence was identical to the active site sequence in *L. major* GP63-1 and is well conserved in GP63 sequences from all other *Leishmania* species. The active site sequence conformed to the His-Glu-Xxx-Xxx-His consensus sequence characteristic of other zinc metalloproteinases (Chaudhuri *et al.* 1989; Bouvier *et al.* 1989; Jongeneel *et al.* 1989).

Figure 7. Amino acid sequences encoded by *Leishmania* gp63 genes.

Lm (#6), *L. major* GP63-6; ***Lmm (C1)***, *L. mexicana* (C1) GP63 (Medina-Acosta *et al.* 1993); ***Lc (C1)***, *L. chagasi* (C1) GP63 (Ramamoorthy *et al.* 1992); ***Lg (N)***, *L. guyanensis* (N) GP63 (Steinkraus and Langer, 1992); ***Lm (#1)***, *L. major* GP63-1 (Button and McMaster, 1988; Button and McMaster, 1990); ***Ld***, *L. donovani* GP63 (Webb *et al.* 1991); ***Lc (L1)***, *L. chagasi* (L1) GP63 (Ramamoorthy *et al.* 1992) and *L. chagasi* GP63 (Miller *et al.* 1990); ***Lc (S1)***, *L. chagasi* (S1) GP63 (Ramamoorthy *et al.* 1992); ***Lg (Y, X, K, and Z)***, *L. guyanensis* cDNA clones encoding GP63 (Steinkraus and Langer, unpublished data). The alignment shows the amino acids using the standard single-letter code. Dots represent identical residues and dashes represent gaps introduced for optimal alignment of the sequences. Amino acids identical for all predicted proteins are indicated with an asterisk (*). Conserved amino acids are also indicated (^). The consensus zinc binding domain (residues 263-268, *Lm #6*) is overlined. The proposed leader signal sequence cleavage site between Ala-39 and His-40 and the proposed pro-peptide cleavage site between Val-99 and Val-100 are indicated (•). The cleavage site for glycosyl-phosphatidylinositol anchor attachment between Asn-577 and Thr-578 for *Lm (#1)* is underlined. Asparagine residues involved in possible *N*-linked glycosylation are highlighted in bold type (**N**). Separation of GP63 proteins into two classes begins on page 41 with *Lm (#6)* heading one class and *Lm (#1)* heading the other class.


Lm (#6) MSVDSSSTHRRRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGALQHRCVHDAMQARVRQSVADHHKAPGAVSAVGLPYVTLDAAHTAAAADPRP--GSAR 98
Lmm (C1) .....H.....P.....P....I.....L....AQRM..S.....SVVPVEN.STL.YSLSDSTSP 100
Lc (C1) .....H.S.....IA.....V....I.....R..T.....---.....R..--...P 95
Lg (N) ..R.R.....S.....IGF....--LVM...A..V..Q.AG--.H.I..RL....L....QQRSV.A.F..L.....STGTISS.HTV.WALADSTSP 96
Lm (#1) .....--.... 98
Ld .....H.S.....IA.....V....I.....R..T.....S....----------.A.P 85
Lc (L1) .....H.S.....IA.....V....I.....R..T.....---.....R..--...P 95
Lc (S1) .....H.S.....IA.....V....I.....R..T.....---.....R..--...P 95
Lg (Y) .PLN.....S.....V.AAL.....A.TP...I..KL.....D.ATHRRMP.S.....I....----------.D 85
Lg (X) .PLN.....S.....V.AAL.....A.TP...I..KL.....D.A.HRRMP.S.....I....----------.D 85
Lg (K) ..RDR.....S.....M.....--LVM...A..V..Q.AG--.H.I..RL....L....QQRRP..S...L.....ST.PISS.H.V.WALADSTSP 96
Lg (Z) .PLN.....S.....V.AAL.....A.TP...I..KL.....D.A.HRRMP.S.....IA...----------.D 85
SVVRDVNWGALRIAVSTEDLTDPAYHCARVGQRVNNHAGAIVTCTAEDILTNEKRDILVKHLIPQAVQLHKERLKVQQVQGKWKVTDMVGEICGDFKVPQ 198
Lmm (C1) G...AA.....V...A.....Q.....D.....D....T.....V...L...R.....R.....G.ADV.....P 200
Lc (C1) T...AA.....A.....D.....Y.....L...T.....R...D..N..G..D.....P 195
Lg (N) ..A.AAD..T.....A.....G...T.....N.E.....V..E.....SY.....L...A.....R....S....G.T.S.....S..T 196
Lm (#1) .....H.KD.....T.....D..... 198
Ld T...AA.....S.....ISTRD.RFAI.....D.....Y.....L...T.....R...D.....G.GN....H....P 185
Lc (L1) T...AA.....HIKRRL.GVDI.....D.....L...T.....R...D.....G.GDDV.S.....P 195
Lc (S1) T...AA.....HIKRRL.GVDI.....D.....L...T.....R...D.....G.GDDV.S.....P 195
Lg (Y) T.A.AAD..T.....A.....D...T.....S...DE.....V..E.....SY.....L...A.....R....T....G.T.DV..K....E 185
Lg (X) T.A.AAD..T.....A.....D...T.....S...DE.....V..E.....SY.....L...A.....R....T....G.T.S.....S..T 185
Lg (K) ....AGD..T...Y..Y.....D.Y.SY...LID..D...DI.E.K....DA.....NV.LR..L...VD....KR...S....G.E.DV..E....E 196
Lg (Z) T.A.AAD..T.....A.....D...T.....S...DE.....V..E.....SY.....L...A.....R....T....G.T.S.....S..T 185
AHITEGFSNTDFVMYVASVPSEEGVLAWATTCQTFSDGHPAVGVINIPAANIASRYDQLVTRVVTHEMAHALGFSGPFFEDARIVANVSNVRGKNFDVPV 298
Lmm (C1) E.....VT.....L.....S.....V.P.....V....T..GAVG..QE.PHL.R.D.N.S. 300
Lc (C1) .....V.....-V....T..TEILL.TQMM.I...D.N.S. 294
Lg (N) ..L.A.VT.A...L.....P.....V...D.....V.....GA.....V.....ST..KS.G..KS.T.L...P.AA.. 296
Lm (#1) .....P..... 298
Ld ....D.L.....GD.....V.....VV..R....LESI....H.D..... 285
Lc (L1) ....D.L.....V.....VG...G...LESI....H.D..... 295
Lc (S1) ....D.L.....A...V.....VG...G...LESI....H.D..... 295
Lg (Y) R.VAK.V..A...L.....P.....V...D.....V.....GT..T....V.....ST..KS.G..KS.T.L...P.AA.. 285
Lg (X) ..L.A.V..A...L.....P.....V...D.....V.....GT..T....V.....ST..KSTGV.M..TG....P.AA.. 285
Lg (K) ..V.V....I...L.....P..M...VI..V.P..R.....H.R.P...IMV.T.A..IT.....DLTV..EMQ.IDE...L..R.YY... 296
Lg (Z) ..L.A.V..A...L.....P.....V...D.....V.....GT.....V.....ST..KSTGV.M..TG....P.AA.. 285
INSSTAVAKAREQYGCDTLEYLEVEDQGGAGSAGSHIKMRNAQDELMAPAAAAGYYTALTMAILQDLGFYQADFSKAEVMPWGQNAGCAFLTNKCMEQSV 398
Lmm (C1) .T...V.....NS.....I.....K.....S.....VF.....E....R.V.....SE...AKN. 400
Lc (C1) .....I.....K.....S.....F.....R.....SE....RNI 394
Lg (N) .....V.....P.....S.....K.....S.....VFE.....K...T.....R..S.D...K....NNI 396
Lm (#1) .....F..... 398
Ld .....G.....M.....SD....S.....F.....E....R.....SE....DGI 385
Lc (L1) .....I.....S.....F.....R.....SE....RNI 395
Lc (S1) .....I.....S.....F.....R.....SE....RNI 395
Lg (Y) .....V.....P.....S.....L.G...K.....S.....VFE.....A.....R..S.D...K....NNI 385
Lg (X) .....V.....P.....S.....L.G...K.....S.....VFE.....K...A.....R..S.D...K....NNI 385
Lg (K) L..P.V.....AS.SF..L..T..TST....L.G...K.....VT....S.....V.....R.V.RL.....AEYSS.D.I.Q....RNI 396
Lg (Z) .....V.....DE...P.....S.....L.G...K.....S.....VFE.....A.....R..S.D...K....NNI 385

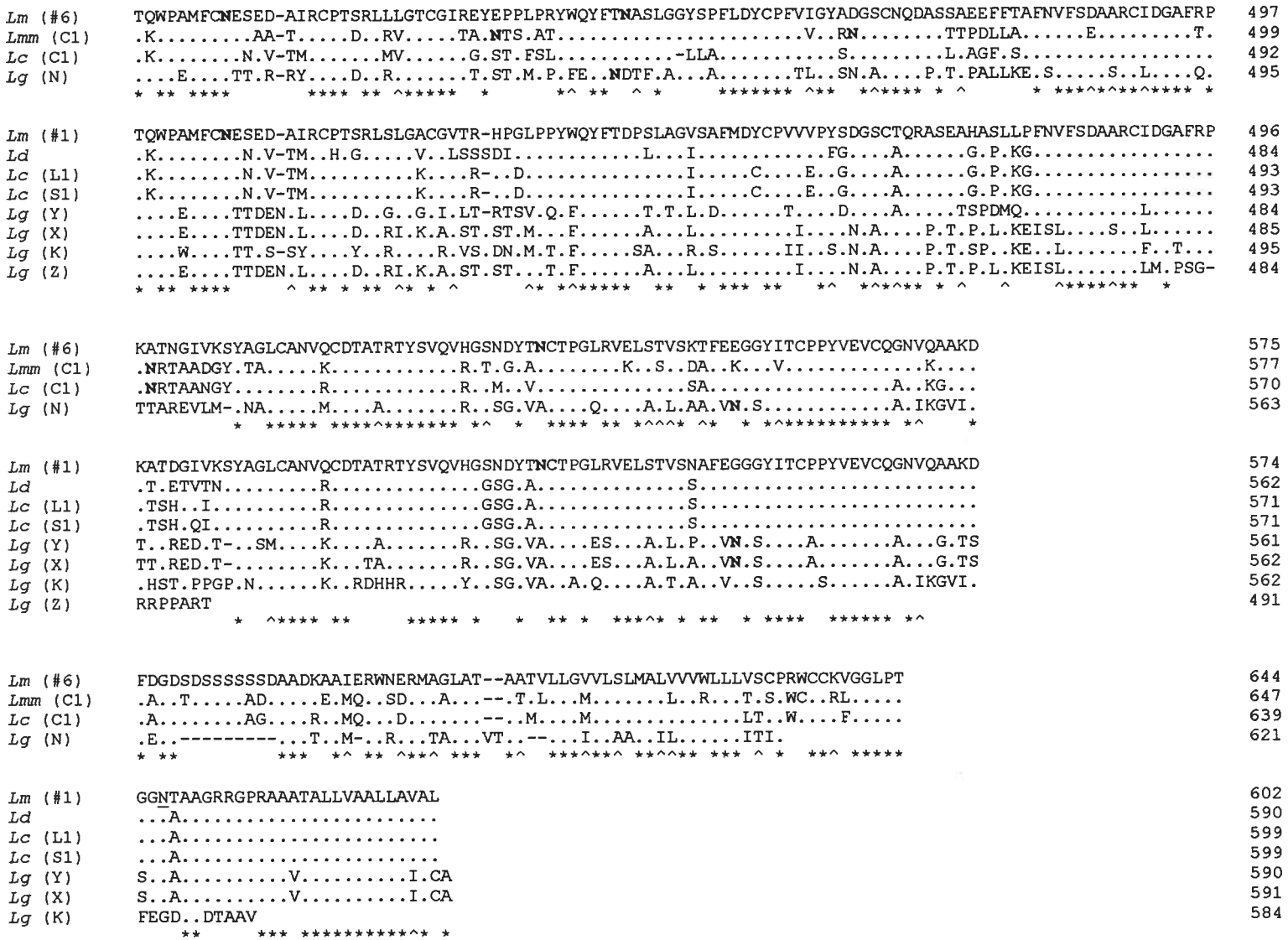
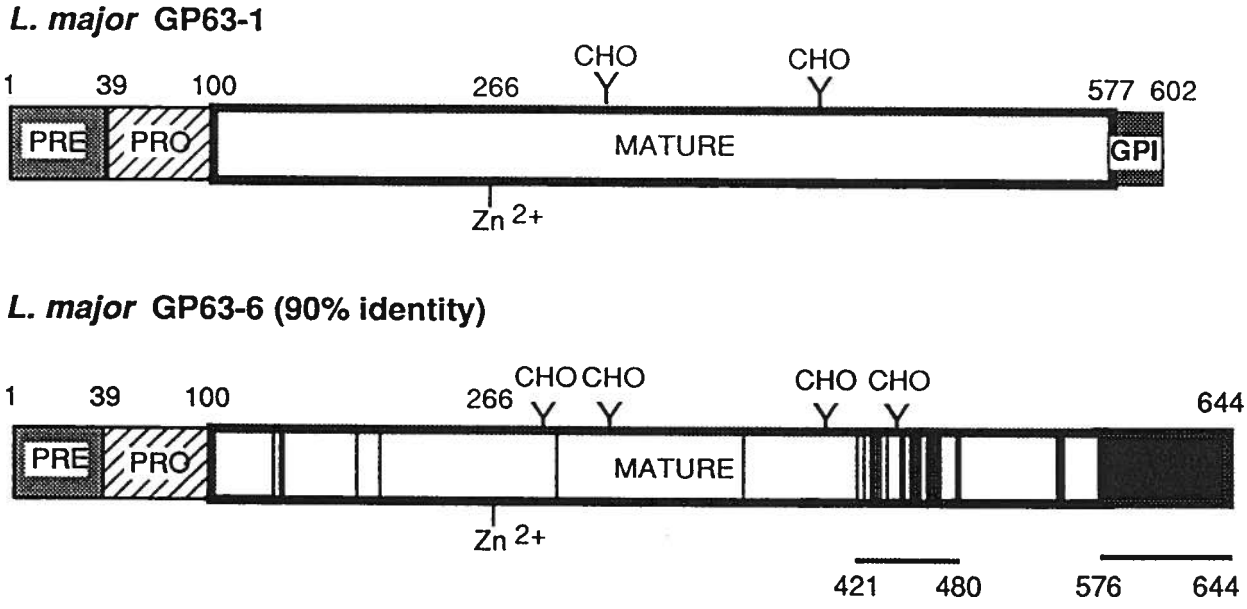

Lm (#6) TQWPAMFCNESED-AIRCPTSRLLLGTCGIREYEPPLPRYWQYFTNASLGGYSPFLDYCPFVIGYADGSCNQDASSAEEFFTAFNVFSDAARCIDGAFRP 497
Lmm (C1) .K.....AA-T.....D..RV.....TA.NTS.AT.....V..RN.....TTPDLLA.....E.....T. 499
Lc (C1) .K.....N.V-TM.....MV.....G.ST.FSL.....-LLA.....S.....L.AGF.S..... 492
Lg (N) ....E....TT.R-RY....D..R.....T.ST.M.P.FE..NDTF.A...A.....TL..SN.A....P.T.PALLKE.S.....S..L....Q. 495
Lm (#1) TQWPAMFCNESED-AIRCPTSRLSLGACGVTR-HPGLPPYWQYFTDPSLAGVSAFMDYCPVVVPYSDGSCTQRASEAHASLLPFNVFSDAARCIDGAFRP 496
Ld .K.....N.V-TM..H.G.....V..LSSSDI.....L...I.....FG....A.....G.P.KG..... 484
Lc (L1) .K.....N.V-TM.....K....R-..D.....I.....C....E..G....A.....G.P.KG..... 493
Lc (S1) .K.....N.V-TM.....K....R-..D.....I.....C....E..G....A.....G.P.KG..... 493
Lg (Y) ....E....TTDEN.L....D..G..G.I.LT-RTSV.Q.F.....T.T.L.D.....T....D....A.....TSPDMQ.....L..... 484
Lg (X) ....E....TTDEN.L....D..RI.K.A.ST.ST.M...F.....A...L.....I....N.A....P.T.P.L.KEISL....S..L..... 485
Lg (K) ....W....TT.S-SY....Y..R....R.VS.DN.M.T.F.....SA...R.S.....II..S.N.A....P.T.SP..KE..L.....F..T... 495
Lg (Z) ....E....TTDEN.L....D..RI.K.A.ST.ST...T.F.....A...L.....I....N.A....P.T.P.L.KEISL.....LM.PSG- 484
Lm (#6) KATNGIVKSYAGLCANVQCDTATRTYSVQVHGSNDYTNCTPGLRVELSTVSKTFEEGGYITCPPYVEVCQGNVQAAKD 575
Lmm (C1) .NRTAADGY.TA.....K.....R.T.G.A.....K..S..DA..K...V.....K.... 577
Lc (C1) .NRTAANGY.....R.....R..M..V.....SA.....A..KG... 570
Lg (N) TTAREVLM-.NA.....M....A.....R..SG.VA....Q....A.L.AA.VN.S.....A.IKGVI. 563
Lm (#1) KATDGIVKSYAGLCANVQCDTATRTYSVQVHGSNDYTNCTPGLRVELSTVSNAFEGGGYITCPPYVEVCQGNVQAAKD 574
Ld .T.ETVTN.....R.....GSG.A.....S..... 562
Lc (L1) .TSH..I.....R.....GSG.A.....S..... 571
Lc (S1) .TSH.QI.....R.....GSG.A.....S..... 571
Lg (Y) T..RED.T-..SM....K....A.....R..SG.VA....ES...A.L.P..VN.S....A.....A...G.TS 561
Lg (X) TT.RED.T-.....K...TA.....R..SG.VA....ES...A.L.A..VN.S....A.....A...G.TS 562
Lg (K) .HST.PPGP.N.....K..RDHHR.....Y..SG.VA..A.Q....A.T.A..V..S.....S.....A.IKGVI. 562
Lg (Z) RRPPART 491
Lm (#6) FDGDSDSSSSSSDAADKAAIERWNERMAGLAT--AATVLLGVVLSLMALVVVWLLLVSCPRWCCKVGGLPT 644
Lmm (C1) .A..T.....AD.....E.MQ..SD...A...--.T.L...M.....L..R...T.S.WC..RL..... 647
Lc (C1) .A.....AG....R..MQ...D.....--..M....M.....LT..W....F..... 639
Lg (N) .E..---------...T..M-..R...TA...VT..--...I..AA..IL.....ITI. 621
Lm (#1) GGNTAAGRRGPRAAATALLVAALLAVAL 602
Ld ...A..... 590
Lc (L1) ...A..... 599
Lc (S1) ...A..... 599
Lg (Y) S..A.....V.....I.CA 590
Lg (X) S..A.....V.....I.CA 591
Lg (K) FEGD..DTAAV 584

Figure 8. Predicted features of *L. major* GP63-6 and *L. major* GP63-1.

A comparison of the predicted proteins encoded by *L. major gp63-6* and *L. major gp63-1* is shown. The main regions of the predicted protein are indicated: **PRE**, prepeptide region; **PRO**, propeptide region; **MATURE**, mature protein; **GPI**, carboxy terminus removed for GPI attachment. Also indicated are the zinc binding domains (Zn^{2+}) and possible N-linked glycosylation sites (CHO). Vertical lines (|) indicate amino acid differences. A cluster of amino acid differences is indicated between residues 421 and 480 in addition to the different carboxy termini of the two GP63s.



The predicted protein encoded by *L. major gp63-6* contains five potential *N*-linked glycosylation sites. Asparagine residues involved in possible *N*-linked glycosylation which conform to the Asn-Xxx-Ser/Thr consensus sequence (Marshall, 1972) were found at positions 285, 300, 407, 443, and 535 (Fig. 7) in *L. major* GP63-6. The predicted sequence of the *L. major* GP63-1 protein contains only three potential *N*-linked glycosylation sites (position 300, 407, and 534; Fig. 7), all three of which are conserved in *L. major* GP63-6. However, asparagines at position 535 in *L. major* GP63-6 and at position 534 in *L. major* GP63-1 (Fig. 7) are followed by a cysteine that would likely be involved in disulphide bonds suggesting that *N*-linked glycosylation at these sites is unlikely.

Interestingly, amino acid differences between the two proteins were found to start two amino acids before the asparagine at position 577 in *L. major* GP63-1 (see Fig. 7 and 8). The asparagine at position 577 (*L. major* GP63-1) has been identified as the GPI anchor attachment site with amino acids 578-602 removed in the mature GPI-linked protein (Schneider *et al.* 1990). In contrast to *L. major* GP63-1, *L. major* GP63-6 encodes a different carboxy terminus that is 41 amino acids longer than the *L. major* GP63-1 carboxy terminus. In addition to the different carboxy termini, an internal region of differences between the two *L. major* GP63s was found between positions 421-480 (Fig. 7 and 8). Within this region only five of twenty-eight amino acid differences were shown to be conserved. Identity was found to resume after position 480 until the divergent carboxy termini (position 576 - end).

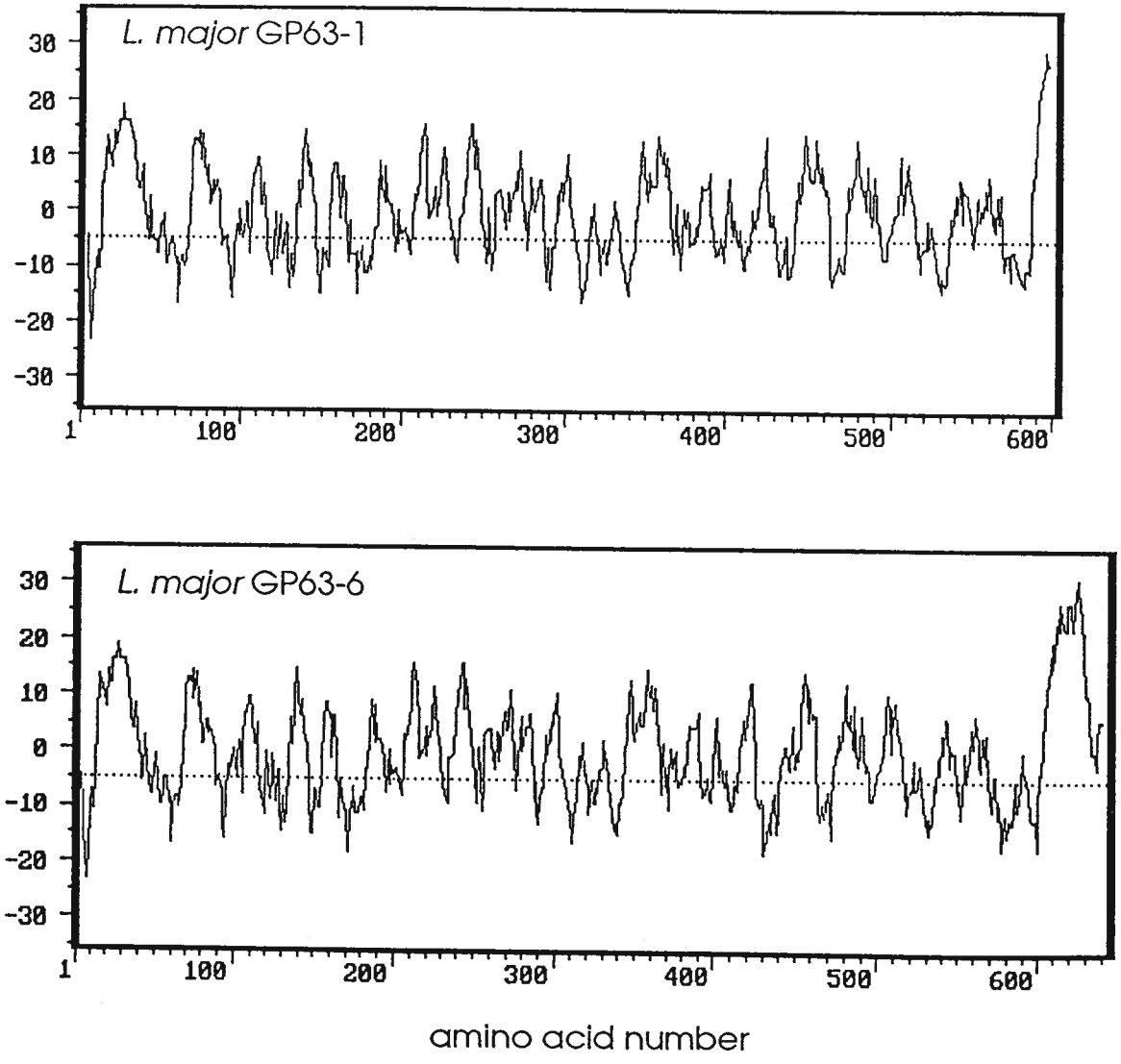
Comparison of the two *L. major* GP63 proteins with those from other *Leishmania* species suggested that GP63 could be grouped into two classes based on the sequence and length of the carboxy terminus. Thus *L. major* GP63-6 is characteristic of one class of GP63 with an extended carboxy terminus while *L. major* GP63-1 is characteristic of the other class of GP63 known to contain a GPI signal sequence. Figure 7 shows GP63 sequences separated into the two classes characterized by *L. major* GP63-6 and *L. major* GP63-1 after amino acid #398 [*Lm* (#6) sequence]. The extended carboxy terminus of *L. major* GP63-6 shared a high degree of identity with *L. mexicana (C1)* GP63 and *L. chagasi (C1)* GP63. The *L. guyanensis (N)* GP63 protein was also found to contain a similar carboxy terminus although it is 23 amino acids shorter than *L.*

major GP63-6 (621 vs. 644 residues). In contrast to GP63 proteins with the extended carboxy termini, the carboxy terminus of the GPI-linked *L. major* GP63 was found to share a high degree of identity with *L. donovani*, *L. chagasi (L1)*, *L. chagasi (S1)*, and *L. guyanensis (K, X, and Y)* forms of GP63 making up the second class of GP63 proteins.

In further support of the grouping of GP63 proteins into two different classes, the internal region of divergence between the two *L. major* sequences (position 421-480, Fig. 7) was shown to be fairly well conserved within the respective classes. The region at positions 421-480 in *L. major* GP63-6 was similar to the corresponding regions in *L. mexicana (C1)* GP63, *L. chagasi (C1)* GP63, and to a lesser degree, *L. guyanensis (N)* GP63. Similarly, region 421-480 in *L. major* GP63-1 was found to be similar to the corresponding regions of *L. donovani* GP63, *L. chagasi (L1)* GP63, *L. chagasi (S1)* GP63, and to a much lesser degree, *L. guyanensis (Y, X, K, and Z)* GP63s.

Hydropathy plot analysis by computer using the method of Klein, Kanehisa, and DeLisi (Klein *et al.* 1985) was carried out to compare hydrophilic and hydrophobic regions and to predict possible transmembrane spanning regions of *L. major* GP63-6 and *L. major* GP63-1. Comparison of the hydropathy plots for *L. major* GP63-6 and *L. major* GP63-1 is shown in Figure 9. The two proteins were shown to be similar in the location of hydrophilic and hydrophobic regions. Hydropathy plot analysis of *L. major* GP63-6 predicted the hydrophobic series of amino acids at positions 611-630 (VLLGVVLSLMALVVVWLLLV) (Fig. 7) near the carboxy terminus to encode a possible transmembrane spanning domain. A hydrophobic region at positions 586-602 (RAAATALLVAALLAVAL) (Fig. 7) in the *L. major* GP63-1 carboxy terminus was also predicted to be a transmembrane region, however, this sequence encompasses the final 17 amino acids of the 25 amino acid carboxy terminus known to be removed from the protein for attachment of the GPI anchor in this GP63 protein (Schneider *et al.* 1990).

Figure 9. Hydropathy plot comparison of *L. major* GP63-1 and *L. major* gp63-6. Amino acid positions are indicated on the X-axis. Regions above the midpoint line at position -5 on Y-axis indicate hydrophobic regions. Regions below the midpoint line indicate hydrophilic regions.



B. EXPRESSION OF L. MAJOR gp63-6 AND L. MAJOR gp63-1

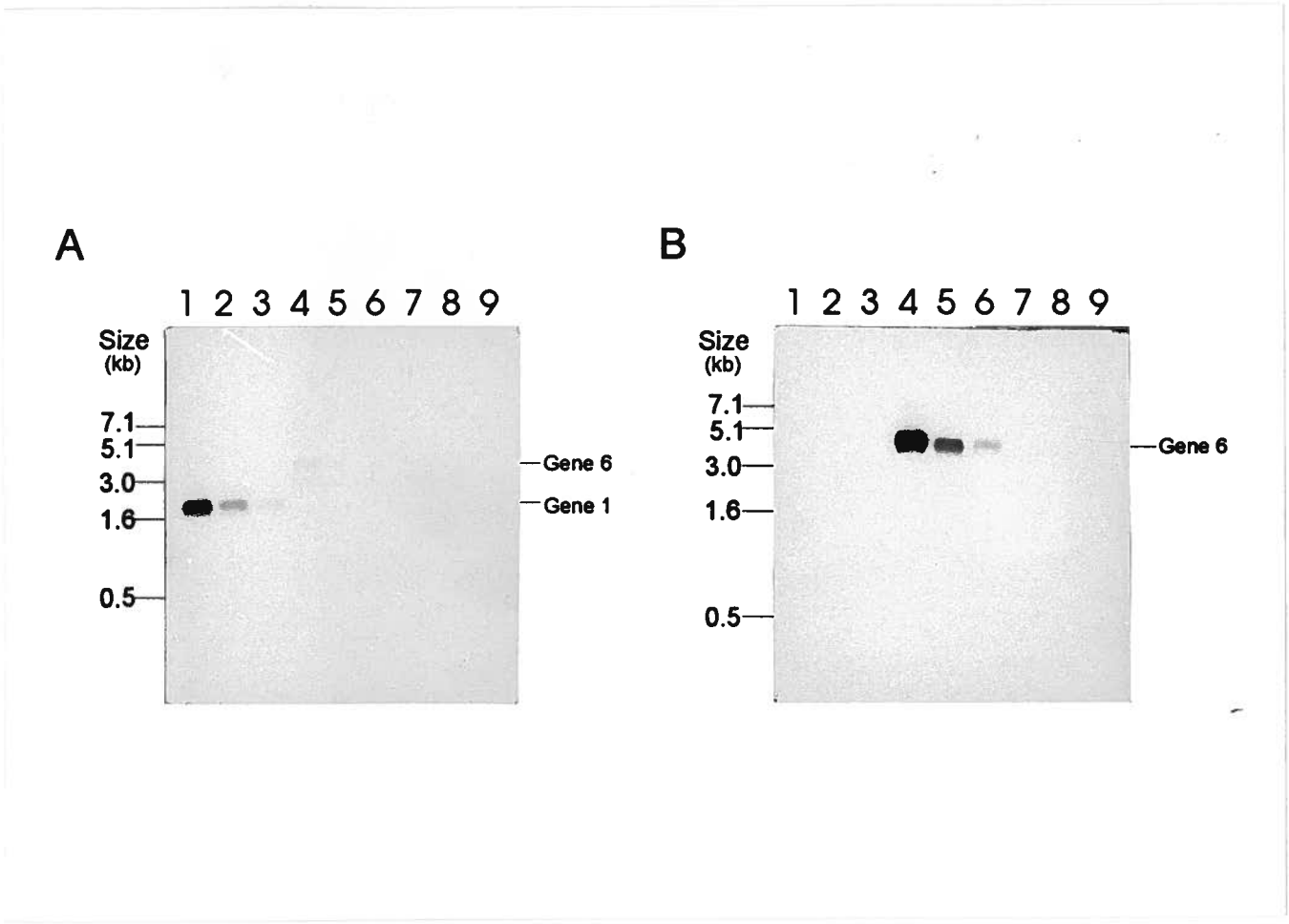
Previous Northern blot analysis of gp63 expression in promastigotes and amastigotes determined gp63 to be expressed in both amastigotes and promastigotes (Button *et al.* 1989). Northern analysis in this case used a hybridization probe common to both *L. major gp63-1* and *L. major gp63-6*. To investigate the specific expression of *L. major gp63-1* and *gp63-6* in the two life stages of *Leishmania*, Northern blot analysis was carried out using hybridization probes designed to be specific to the different 3' ends of *L. major gp63-1* and *gp63-6*. (See Fig. 3 for location of primers used in PCR to generate the *L. major gp63-1* and *L. major gp63-6* specific hybridization probes).

To determine and verify the specificity of the *L. major gp63-1* and *L. major gp63-6* hybridization probes, Southern blot analysis of plasmids containing either *L. major gp63-1* or *L. major gp63-6* was carried out with the two hybridization probes. Results indicated some cross-hybridization with the pBluescript vector as well as with gp63 DNA of the other gene for both hybridization probes despite stringent wash conditions (0.1% SSC, 0.1% SDS, 65°C) (data not shown). In an attempt to prevent cross-hybridization, an excess of unlabeled linearized plasmid DNA containing the other gene was added to the specific hybridization probes. Five μg of unlabeled linearized plasmid DNA containing *L. major gp63-1* was added to the *L. major gp63-6* specific probe. Similarly, 5 μg of unlabeled linearized plasmid DNA containing *L. major gp63-6* was added to the *L. major gp63-1* specific probe. Southern blot analysis was carried out to verify whether or not this procedure would effectively prevent cross-hybridization. Figure 10 shows results of Southern analysis of plasmid DNA containing *L. major gp63-1* or *L. major gp63-6*. A 2.2 kb *Eco* RI insert containing *L. major gp63-1* along with 0.2 kb of 5' and 0.2 kb of 3' flanking regions was excised from the plasmid pBS10Rb.1 (Button *et al.* 1988). Similarly, a 4.1 kb *Sal* I insert containing *L. major gp63-6* along with 1.1 kb of 5' and 1.0 kb of 3' flanking regions was excised from pLGS6.1. The pBluescript KS+ vector was linearized with *Xho* I and also used in the analysis. Southern hybridization with the *L. major gp63-1* specific probe containing 5 μg of unlabeled linearized pLGS6.1 (gene 6) indicated the expected hybridization with the 2.2 kb *Eco*

RI fragment containing *L. major gp63-1* (lanes 1-3, Fig. 10A). A very faint 4.1 kb signal was detected in the lane containing 50 ng of pLGS6.1 (lane 4, Fig. 10A) indicating a very low level of cross-hybridization of the *L. major gp63-1* specific probe with *L. major gp63-6* DNA. No cross-hybridization was detected in lanes containing 5 ng or 0.5 ng of pLGS6.1 plasmid DNA (lane 5 and 6, Fig. 10B). Southern analysis with the *L. major gp63-6* specific probe containing 5 μg of unlabeled linearized pBS10Rb.1 (gene 1) shows the expected hybridization with the 4.1 kb *Sal* I fragment containing *L. major gp63-6* (lanes 4-6, Fig. 10B). No cross-hybridization was detected with *L. major gp63-1* DNA (lanes 1-3). These results therefore indicate that the *L. major gp63-1* and *gp63-6* specific probes are specific for their respective genes and show little or no cross-hybridization when used with 5 μg of unlabeled linearized plasmid DNA containing the other *L. major* gp63 gene to compete out any possible cross-hybridization. Previous Northern blot results of *L. major* gp63 expression estimated that gp63 transcripts account for 0.05% of total RNA or 1% of the mRNA (Button *et al.* 1989). Therefore using 5 μg of unlabeled plasmid DNA in Northern blot analysis of 5 μg of total RNA would represent a 2000 fold excess of DNA over all gp63 transcripts and would therefore effectively prevent any potential cross-hybridization with transcripts from the other gene in Northern analysis.

Figure 10. Southern blot analysis of plasmid DNA containing *L. major gp63-1* and *L. major gp63-6*.

Plasmid DNA; pBS10Rb.1 (containing *L. major gp63-1*), pLGS6.1 (containing *L. major gp63-6*), and pBluescript KS+ was separated on 0.75% agarose gels before blotting. Lanes 1-3: 50 ng, 5 ng, and 0.5 ng of pBS10Rb.1 digested with *Eco* RI respectively. Lanes 4-6: 50 ng, 5 ng, and 0.5 ng of pLGS6.1 digested with *Sal* I respectively. Lanes 7-9: 50 ng, 5 ng, and 0.5 ng of pBluescript KS+ digested with *Xho* I respectively. **A**: Hybridization with the *L. major gp63-1* specific probe along with 5 μg of unlabeled *Xho* I linearized pLGS6.1. **B**: Hybridization with the *L. major gp63-6* specific probe along with 5 μg of unlabeled *Xho* I linearized pBS10Rb.1. Southern hybridization conditions and probes are described in Materials and Methods. The sizes of DNA markers are indicated on the left in kb.



Northern blot analysis of *L. major* gp63 expression was carried out using total RNA isolated from log and stationary phase *L. major* promastigotes and lesion amastigotes isolated from infected BALB/c mice. Lesion amastigote RNA was found to contain mouse RNA and therefore mouse RNA isolated from spleen leukocytes of noninfected BALB/c mice was included in Northern analysis to serve as a control for amastigote RNA. RNA was separated on a 2.2 M formaldehyde agarose gel before blotting. Duplicate RNA samples and an RNA ladder were included in the gel for ethidium bromide staining. Figure 11 shows the duplicate RNA samples and RNA ladder stained with ethidium bromide. The band corresponding to mouse 28s ribosomal RNA is indicated and can be seen in the amastigote RNA sample preparation (lane A) and the mouse RNA sample (lane L). The three *Leishmania* ribosomal RNAs (Villalba *et al.* 1985) were found to migrate at 2.25, 1.85, and 1.55 kb respectively and can be seen in both promastigote RNA and amastigote RNA samples (lanes PL, PS, and A).

Results of Northern blot analysis of *L. major* and mouse spleen RNA hybridized with the *L. major gp63-1* and *L. major gp63-6* specific probes (containing 5 μg of unlabeled linearized plasmid DNA containing the other gene as described above) are shown in Figure 12. Hybridization with the *L. major gp63-1* specific probe (Fig. 12A) resulted in detection of a predominant 2.6 kb transcript and a minor 4.8 kb transcript in log and stationary phase promastigote RNA (lanes PL, PS). A very low level of hybridization to amastigote RNA was detected at 2.6 kb. In contrast, the *L. major gp63-6* specific probe hybridized to a 3.5 kb transcript in RNA from both promastigote phases as well as the amastigote stage (Fig. 12B, lane PL, PS, and A). A faint smear in the 2.7 kb to 2.9 kb range was also observed in the amastigote RNA. No hybridization was detected in mouse spleen RNA.

The Northern blots which were hybridized with the *L. major gp63-1* or *gp63-6* specific probes (Fig. 12A and B) were stripped and rehybridized with a *Leishmania* β-tubulin probe to verify that the RNA loaded was intact for all Leishmania samples in both blots. Results of this analysis are shown in Figure 12C and D. Consistent with reports of expression of three β-tubulin transcripts in promastigotes of *L. major* (Spithill and Samarus, 1987) three transcripts of 3.4, 2.8, and 2.2 kb were detected in promastigote RNA samples (lanes PL, PS, Fig. 12C and D). Three

transcripts of 2.9, 2.4, and 1.4 kb were detected in the amastigote RNA samples (lane A, Fig. 12C and D). Detection of distinct transcripts in all Leishmania RNA samples in both blots indicates that the RNA was intact for all samples. Hybridization with the β-tubulin probe cannot be used to quantitate RNA loading since expression of β-tubulin in Leishmania is developmentally regulated (Spithill and Samarus, 1987).

These results indicate that at the level of detection by Northern blot analysis, *L. major gp63-1* is expressed as a 2.6 kb transcript in the promastigote life stages while *L. major gp63-6* is constitutively expressed as a 3.5 kb transcript in both the amastigote and promastigote life stages. The minor 4.8 kb transcript detected in log and stationary phase promastigote RNA by the *L. major gp63-1* specific probe may represent an intermediate in the processing of a polycistronic precursor mRNA (Button *et al.* 1989).

Figure 11. Ethidium bromide staining of RNA duplicate samples used in Northern blot analysis.

Two μg of total *L. major* RNA or mouse RNA was loaded per lane on a 1% agarose gel containing 2.2 M formaldehyde and separated by electrophoresis at 50 V for 3 hours. The gel was stained with 0.5 mg ml^{-1} ethidium bromide to visualize the RNA. **PL**, log phase promastigotes RNA; **PS**, stationary phase promastigote RNA; **A**, mouse lesion amastigote RNA; **L**, leukocyte RNA isolated from noninfected mice spleen leukocytes. Promastigote and leukocyte RNA samples were quantitated by spectrophotometer. *L. major* amastigote RNA was quantitated by visual estimation using ethidium bromide staining agarose gel electrophoresis. The position of mouse 28s ribosomal RNA is indicated as are the sizes of RNA markers in kb.

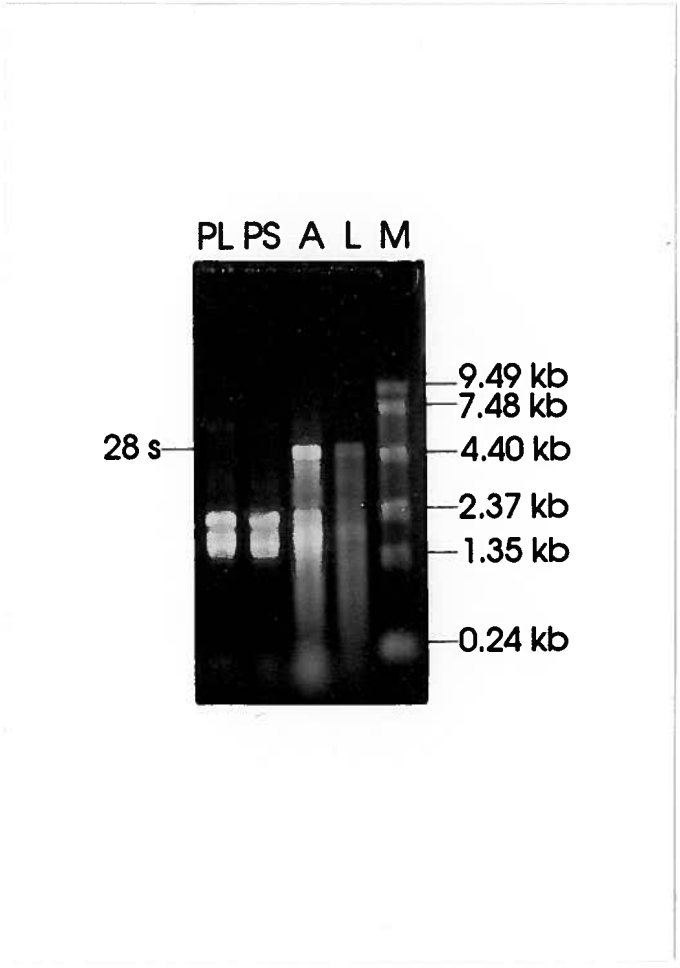
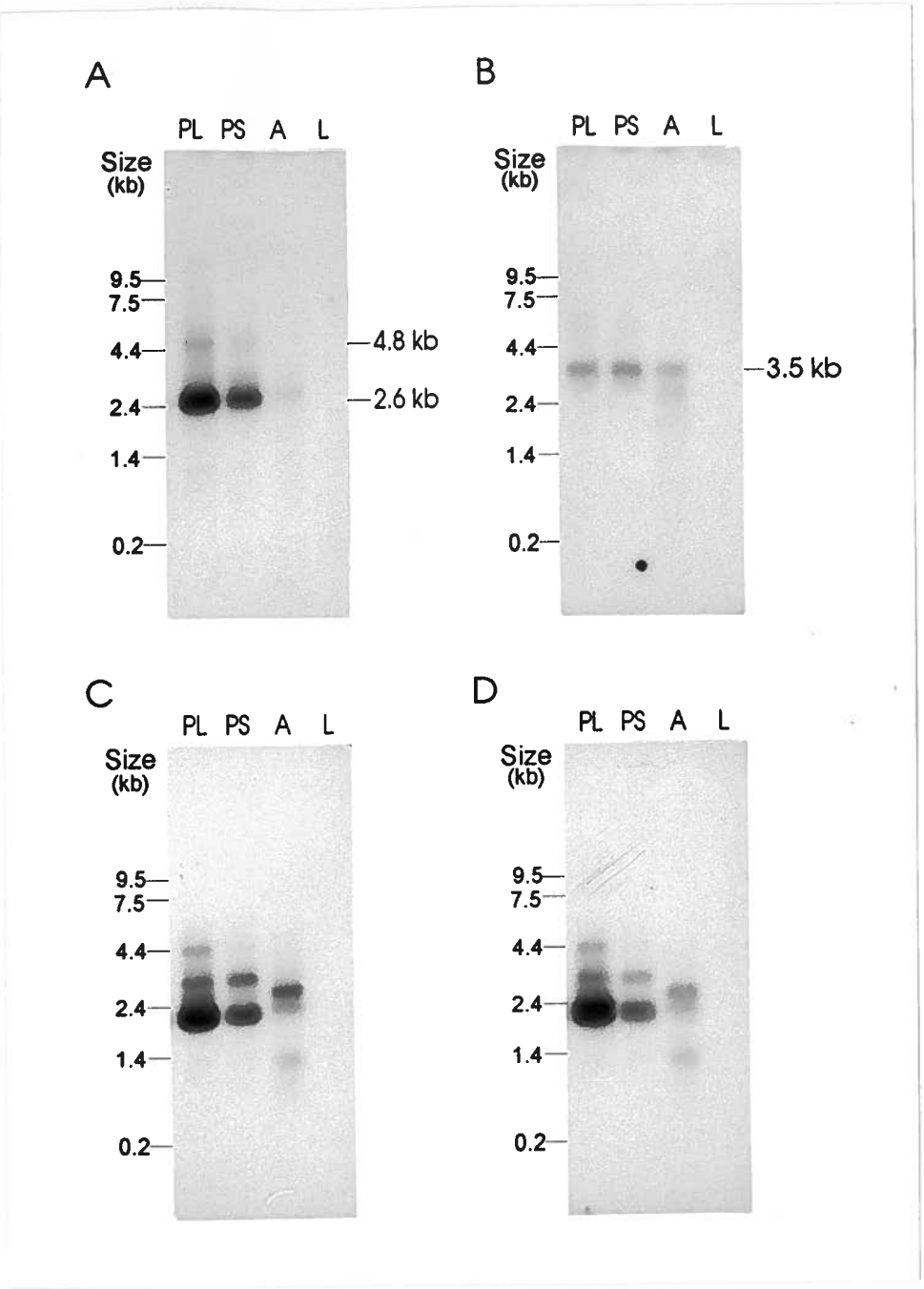


Figure 12. Northern blot analysis of *L. major* gp63 expression.

Five μg of total RNA was loaded per lane and separated on a 1% agarose gel containing 2.2 M formaldehyde at 50 V for 3 hours. Northern hybridization conditions are described in Materials and methods. PL, log phase *L. major* promastigotes RNA; PS, stationary phase *L. major* promastigote RNA; A, *L. major* lesion amastigote RNA; L, mouse leukocyte RNA isolated from noninfected mouse spleen leukocytes. **A**: hybridization with *L. major gp63-1* specific probe and 5 μg of unlabeled linearized pLGS6.1 DNA. **B**: hybridization with *L. major gp63-6* specific probe and 5 μg of unlabeled linearized pBS10Rb.1 DNA. **C**: Blot A stripped and rehybridized with a 0.85 kb *Eco* RI fragment of the *L. major* β-tubulin cDNA clone pP121/4 (Spithill and Samarus, 1987). **D**: Blot B stripped and rehybridized with the 0.85 kb *Eco* RI fragment of the *L. major* β-tubulin cDNA clone pP121/4. The sizes of the RNA markers are indicated in kb. Blots A and B were exposed for 5 days while C and D were exposed overnight.

Figure 12. Northern blot analysis of *L. major* gp63 expression.



C. EXPRESSION OF L. MAJOR GP63-6

There is ample evidence that GP63 is expressed as a GPI linked protein on the surface of promastigotes of all *Leishmania* species investigated to date (Bordier *et al.* 1986; Schneider *et al.*. 1990; Etges *et al.* 1986; Chang and Chang, 1986; Colmer-Gould *et al.* 1985). Sequencing results of the present study showed that the predicted protein encoded by *L. major gp63-6* has a different carboxy terminus than that of the GPI-linked protein encoded by *L. major gp63-1*. This finding raised the question of whether or not *L. major* GP63-6 is a surface expressed membrane protein in promastigotes. To investigate this possibility, another *Leishmania* species, *L. donovani*, was transfected with a plasmid expression vector containing either *L. major gp63-6* or *L. major gp63-1* by electroporation. Surface expression of the *L. major* GP63 proteins was subsequently analyzed by flow cytometry using the anti-GP63 mouse monoclonal antibody #96 [mAb #96, described in Macdonald *et al.* (1994), was raised against *L. major gp63-1* recombinant protein]. *L. donovani* promastigotes were used since mAb #96 does not recognize *L. donovani* GP63 (unpublished results) and therefore the expression of individual *L. major* gp63 genes could be analyzed at a protein level in this species.

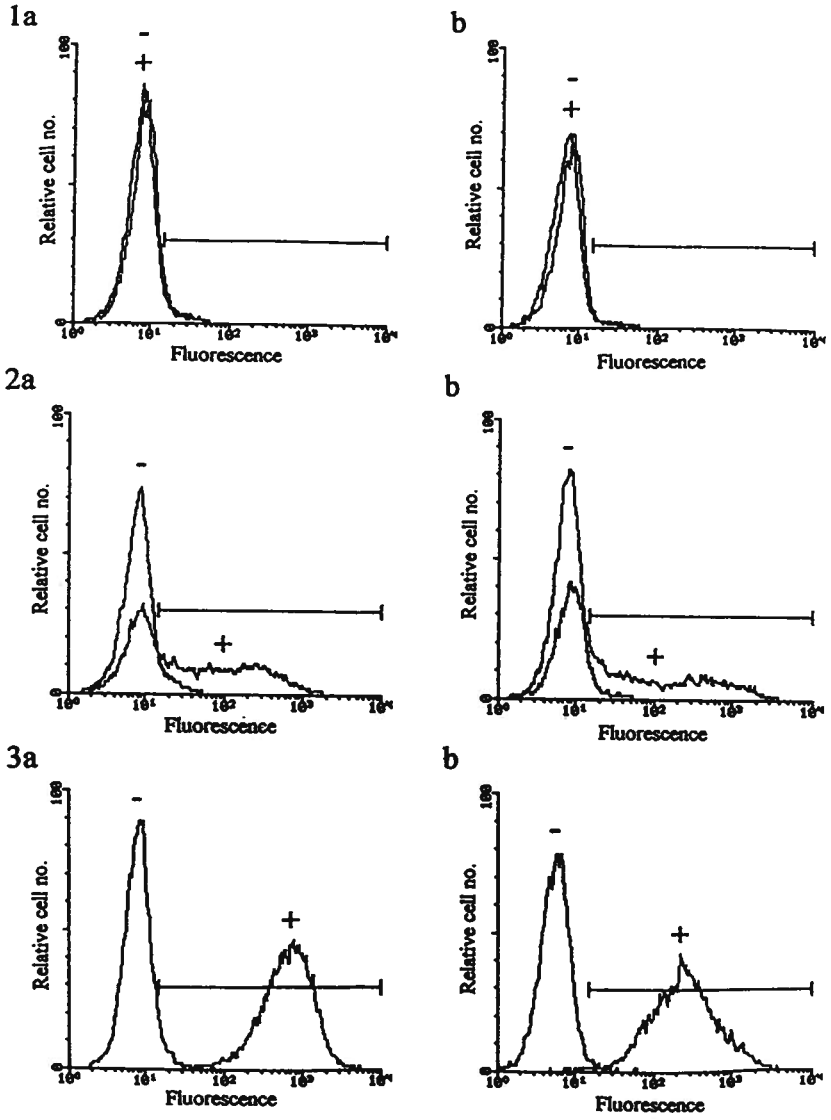
Stable transfection of *L. donovani* promastigotes with expression vector constructs containing *L. major gp63-1* or *L. major gp63-6* was carried out for expression of the two different forms of *L. major* GP63. The two *L. major* gp63 genes were individually cloned into the *Leishmania* expression vector pLEX (described in Webb, 1993) to generate the plasmids pLEXLMGP63-1 or pLEXLMGP63-6. The pLEX vector contains a selectable marker conferring resistance to the antibiotic hygromycin B allowing for selection of transfected *Leishmania* clones. *L. donovani* was transfected with either pLEX, pLEXLMGP63-1, or pLEXLMGP63-6 plasmid DNA by electroporation. Electroporated promastigotes were plated on semi-solid M199 plates containing hygromycin for selection. Drug resistant colonies arising on the plates were picked and expanded in liquid media containing hygromycin and subsequently analyzed for GP63 expression.

Fluorescence flow cytometry analysis with mAb #96 was carried out to study the surface expression of *L. major* GP63 in the *L. donovani* transfectants. The high degree of similarity (90.4%) between the two amino acid sequences predicted by *L. major gp63-1* and *L. major gp63-6* genes indicated the possibility that mAb #96 could also recognize the protein encoded by gene six. Results of FACScan analysis of *L. donovani* clones transfected with either pLEX, pLEXLMGP63-1, or pLEXLMGP63-6 are shown in Figure 13. *L. donovani* transfected with pLEXLMGP63-1 served as positive control cells in FACScan analysis since *L. major gp63-1* is known to be expressed as a surface protein. FACscan analysis of two separate *L. donovani* clones transfected with pLEXLMGP63-1 (containing gene 1) is shown in Figure 13 (2a and b). Promastigotes labeled with mAb #96 [2a (+) and b (+)] showed a 47.5% shift (clone a) or a 42.7% shift (clone b) of fluorescently labeled cells compared to unlabeled cells [2a (-) and b (-)]. FACScan analysis of two *L. donovani* clones transfected with the pLEX vector alone is shown in Figure 13 (1a and b). Results show that the mAb #96 did not recognize endogenous GP63 expressed by *L. donovani* since cells labeled with mAb #96 [1a (+) and b (+)] showed essentially the same distribution as those not labeled [1a (-) and b (-)]. FACScan analysis of two separate *L. donovani* clones transfected with pLEXLMGP63-6 (containing gene 6) is shown in Figure 13 (3a and b). Promastigotes labeled with mAb #96 [3a (+) and b (+)] clearly show a shift in distribution with over 99% of the cells labeled with the antibody compared to unlabeled cells [3a (-) and b (-)]. These results show that the *L. major* specific anti-GP63 mAb #96 recognized GP63 expressed on the surface of *L. donovani* promastigotes transfected with *L. major gp63-6* and therefore strongly suggests that *L. major GP63-6* is expressed as a surface protein in promastigotes.

Figure 13. Fluorescence flow cytometry analysis of *L. donovani* transfected with *L. major gp63-1* and *L. major gp63-6*.

Cells (5 x 10^6) were analyzed by flow cytometry using fluorescence activated cell scanning (FACScan) as described in Materials and Methods. Promastigotes were incubated with monoclonal antibody #96 which recognizes native *L. major* GP63 (+) or with 0.5% BSA/PBS (-) followed by labeling with fluorescein-conjugated goat anti-mouse IgM antibody (10 μg ml^{-1}). Log fluorescence is indicated on the X-axis (arbitrary units) and relative cell number is indicated on the Y-axis. **1a and b**: two separate *L. donovani* clones transfected with pLEX vector. **2a and b**: two separate *L. donovani* clones transfected with pLEXLMGP63-1. **3a and b**: two separate *L. donovani* clones transfected with pLEXLMGP63-6. The ├─┤ represents a marker positioned in the same location for all plots which initially was set to represent 5% of the unlabeled vector transfected control cells (1a -).

Figure 13. Fluorescence flow cytometry analysis of *L. donovani* transfected with *L. major gp63-1* and *L. major gp63-6*.



In an attempt to characterize the GP63 protein expressed by the *L. donovani* pLEXLMGP63-6 transfectants, western blot analysis was carried out with cell lysates of the *L. donovani* transfectants using two different anti-GP63 monoclonal antibodies previously described (Button *et al.* 1991). However to determine whether or not the antibodies described would recognize the two different forms of *L. major* GP63, the two *L. major* gp63 genes were individually transfected into COS-7 cells for subsequent western blot analysis. Expression of the individual forms of GP63 in COS cells was carried out to help determine which antibody would be useful in western blot analysis of the *L. major gp63-6 L. donovani* transfectants. *L. major gp63-1* and *L. major gp63-6* were each individually cloned into the COS cell expression vector PAX 111 (described in Kay *et al.* 1990; Kay and Humphries, 1991) to generate the clones PAXLMGP63-1 and PAXLMGP63-6 respectively. Plasmid DNA was introduced into COS cells by electroporation and cells were grown for 72 hours to allow for expression of the exogenous genes. Lysates of the COS cell transfectants were analyzed by western blot analysis using anti-GP63 antibodies previously described (Button *et al.* 1991).

Western blot analysis of lysates of COS cells transfected with vector only (PAX 111), *L. major gp63-1* (PAXLMGP63-1), and *L. major gp63-6* (PAXLMGP63-6) was carried out using the monoclonal antibody CP3.235. Monoclonal antibody CP3.235 was previously shown to recognize *L. major* and *L. donovani* GP63 (Button *et al.* 1991). Results of this analysis are shown in Figure 14 (1b). Endoglycosidase F treatment of lysates which removes *N*-linked carbohydrate groups was included in western blot analysis to characterize deglycosylated protein. No protein was detected in lysates of vector transfected control cells (lane 1). Lysates of COS cells transfected with PAXLMGP63-1 were found to contain two bands of 64 kDa and 62 kDa before Endoglycosidase F treatment (lane 2 -) and two bands of 64 kDa and 52 kDa after treatment (lane 2 +). The two different sized products could be the result of incomplete processing of the pre-propeptide region of the GP63 protein in COS cells or differential glycosylation. The 64 kDa band before and after Endoglycosidase F treatment (lane 2 - and +) could represent GP63 protein which retained the pre-propeptide region of the protein and was was not glycosylated. The fact that the band retains a molecular weight of 64 kDa following

Endoglycosidase F treatment may support the case for the protein not being glycosylated. Alternatively, the higher molecular weight protein may represent a different glycosylated form of GP63 which may have more bulky carbohydrate groups which may not have been susceptible to Endoglycosidase F treatment. The 62 kDa band before Endoglycosidase F treatment (lane 2 -) is reduced to a 52 kDa band after Endoglycosidase F treatment (lane 2 +). A 67 kDa band was recognized in western blots of lysates of COS cells expressing *L. major gp63-6* (lane 3). Endoglycosidase F treatment had no effect on the apparent molecular weight of the protein (compare - and +, lane 3). This finding could also be the result of incomplete processing of the *L. major* GP63-6 in COS cells with no *N*- linked glycosylation or this form of the protein may not be susceptible to deglycosylation. The results were confirmed by repeated analysis (data not shown). These results therefore indicate that the monoclonal antibody CP3.235 recognizes both *L. major* GP63-1 and *L. major* GP63-6 synthesized in COS cells.

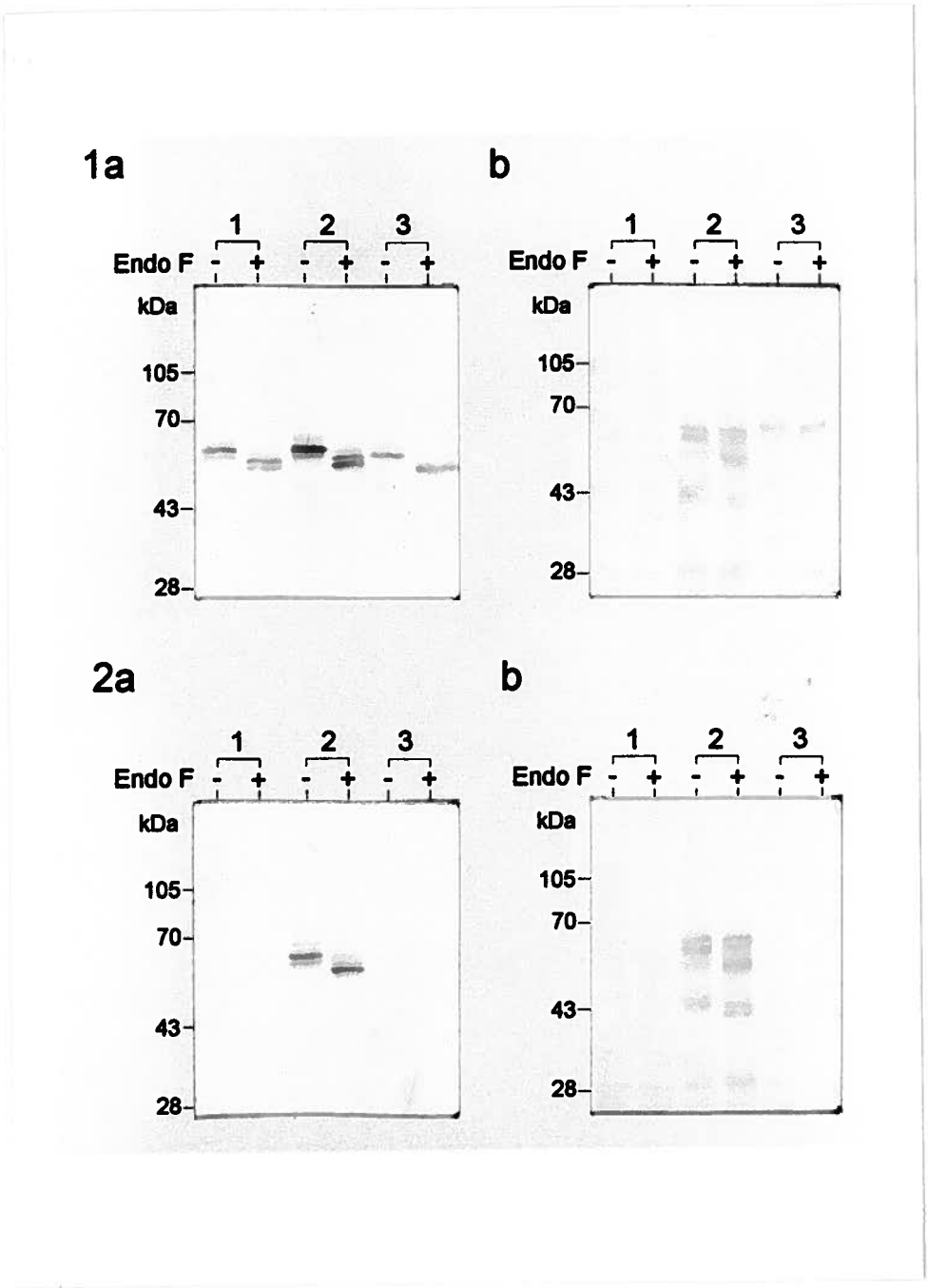
Western blot analysis of lysates of transfected COS cells using monoclonal antibody CP3.139 was carried out to determine whether this antibody would recognize *L. major* GP63-6. The CP3.139 antibody was previously shown to recognize *L. major* GP63 but not *L. donovani* GP63 (Button *et al.* 1991). Results of western analysis are shown in Figure 14 (2b). No protein was detected by the CP3.139 antibody in lysates of the vector transfected control cells (lane 1). *L. major* GP63-1 expressed by COS cells transfected with PAXLMGP63-1 was detected with an apparent molecular weight of 64 kDa and 62 kDA before Endoglycosidase F treatment (lane 2 -) and 64 kDa, 62 kDa, and 52 kDa after treatment (lane 2 +). As in the CP3.235 blot (Fig. 1b), the 64 kDa and 62 kDa bands in the Endoglycosidase F lane (lane 2 +) may represent unprocessed GP63 and/or glycosylated GP63 not susceptible to Endoglycosidase F treatment. Other minor bands are detected in these lanes which could represent degraded forms of GP63. The CP3.139 antibody did not detect any protein in lysates of PAXLMGP63-6 transfected COS cells (lane 3) and therefore does not recognize the protein encoded by *L. major gp63-6*. The determinant for CP3.139 was previously mapped to the region between amino acids 419 and 513 of the GP63 protein encoded by *L. major gp63-1* (see Fig. 7, *Lm (#1)*) (Linda Button, UBC, unpublished data). Comparison of the predicted amino acid sequences of *L. major* GP63-1 and GP63-6 (Fig.

7) indicates this region is quite different in the two predicted proteins. Antigenic differences in this region is therefore the most likely explanation why the CP3.139 antibody does not recognize the *L. major* GP63-6 protein.

Figure 14. Western blot analysis of *L. donovani* and COS-7 cells transfected with *L. major gp63-1* and *L. major gp63-6.*

Lysates of *Leishmania* promastigote (1.5×10^6 cells) or COS cells (1×10^5 cells) were digested with (+) or without (-) Endoglycosidase F overnight. Proteins were denatured in the presence of DTT by boiling samples and separated by SDS polyacrylamide gel electrophoresis on an 8% separating gel. Antibodies and western blot analysis is described in Materials and Methods. The position of prestained high molecular weight protein standards is indicated in kDa on the left of each blot. *Leishmania* extracts (1a and 2a): Lanes 1, *L. donovani* transfected with pLEX vector (clone a from Fig. 13: 1a); Lanes 2, *L. donovani* transfected with pLEXLMGP63-1 (clone a from Fig. 13: 2a); Lanes 3, *L. donovani* transfected with pLEXLMGP63-6 (clone a from Fig. 13: 3a). COS cell extracts (1b and 2b): Lanes 1, COS cells transfected with the PAX 111 vector; Lanes 2, COS cells transfected with PAXLMGP63-1; Lanes 3, COS cells transfected with PAXLMGP63-6. **1a and b**: western blot analysis with mouse monoclonal antibody CP3.235 hybridoma supernatant diluted 1:4. **2a and b**: western blots analysis with mouse monoclonal antibody CP3.139 hybridoma supernatant diluted 1:4.

Figure 14. Western blot analysis of *L. donovani* and COS-7 cells transfected with *L. major gp63-1* and *L. major gp63-6.*



Western blot analysis of lysates of *L. donovani* transfectants using monoclonal antibody CP3.139 was carried out as a comparison to the CP3.139 western blot of COS cell transfectants. Results are shown in Fig. 14 (2a). No protein was detected in lysates of control *L. donovani* promastigotes transfected with the pLEX vector (lane 1). This confirmed earlier results which indicated that CP3.139 does not recognize endogenous *L. donovani* GP63 (Button *et al.* 1991). Two minor bands of 69 kDa and 62 kDa and a major band of 65 kDa were detected in lysates of *L. donovani* transfected with pLEXLMGP63-1 before treatment of Endoglycosidase F (lane 2 -) while two minor bands of 65 kDa and 61 kDa and a major band of 60 kDa were detected after treatment with Endoglycosidase F (lane 2 +). CP3.139 did not detect any GP63 protein of lysates of the *L. donovani* cells transfected with pLEXLMGP63-6 (lane 3). These results were therefore consistent with the results of the CP3.139 western blot of COS cell transfectants that indicated CP3.139 only recognizes *L. major* GP63-1 but not *L. major* GP63-6 (see Fig. 14: 2b).

Although CP3.235 recognizes endogenous *L. donovani* GP63 (Button *et al.* 1991), there remained the possibility that expression of *L. major* GP63-6 in the pLEXLMGP63-6 *L. donovani* transfectants could result in a band distinguishable from the bands reflecting endogenous GP63 detected in control cell lysates in western blot analysis. To investigate this possibility, western analysis of lysates of the *L. donovani* transfectants was carried out with the CP3.235 antibody with results shown in Figure 14 (1a). Analysis of lysates of the control *L. donovani* promastigotes (transfected with the pLEX vector) indicated that the CP3.235 antibody recognizes two bands with an apparent molecular weight of 64 kDa and 61 kDa before treatment of Endoglycosidase F (lane 1 -) and two bands of 60 kDa and 57 kDa after treatment (lane 1 +). CP3.235 therefore was demonstrated to recognizes two endogenous forms of GP63 in *L. donovani*. Western analysis of lysates of *L. donovani* transfected with pLEXLMGP63-1 containing *L. major gp63-1* shows that CP3.235 recognizes the same sized bands as the vector control transfected cells before and after Endoglycosidase F treatment (compare lane 2: - and + to lane 1: - and +) indicating that *L. major* GP63-1 likely comigrated with one of the endogenous *L. donovani* GP63 proteins. In contrast to results of the gene 1 transfectants, western analysis of *L. donovani* transfected with pLEXLMGP63-6 indicated that CP3.235 recognized only one band of

61 kDa before Endoglycosidase F treatment (lane 3 -) and one band of about 55 kDa after treatment (lane 3 +). Whether or not endogenous *L. donovani* GP63 is present in this band is unclear. The fact that over 99% of these pLEXLMGP63-6 transfected promastigotes were shown to express GP63 recognized by the *L. major* GP63 specific mAb #96 in FACScan analysis (see Fig. 13, 3a) would suggest that the 55 kDa band in lane 3 (+ Endoglycosidase F treatment) is likely the *L. major* GP63-6. Western blots of the other *L. donovani* transfected clones (Fig. 13: 1b, 2b, and 3b) had identical results to those seen in Figure 14 (data not shown).

III. DISCUSSION

GP63 is a highly abundant surface glycoprotein found in *Leishmania* and is estimated to occur at about 500,000 copies per promastigote (Etges *et al.* 1986). GP63 has been characterized as a zinc metalloprotease but the exact enzymatic role of the protein is not known. The fact that genes encoding GP63 are highly conserved across all species of *Leishmania* studied to date suggests functional significance of the protein in the biology of the parasite (Button *et al.* 1989; Webb *et al.* 1990; Miller *et al.* 1990; Steinkraus and Langer, 1992; Medina-Acosta *et al.* 1993). Results presented in this study however indicate that GP63 is encoded by a heterogeneous gene family in *L. major* encoding two very similar yet distinctly different forms of GP63.

In *L. major*, GP63 was previously shown to be encoded by a tandem array of five highly conserved genes and a less conserved sixth gene separated from the tandem repeats (Button *et al.* 1989). Sequencing of the sixth gene indicated that the predicted protein was highly conserved in comparison with the protein encoded by the sequence of *L. major gp63-1* (Button *et al.* 1988; Button and McMaster, 1990). The zinc binding active site characteristic of GP63 and other zinc metalloproteases (Chaudhuri *et al.* 1989; Bouvier *et al.* 1989; Jongeneel *et al.* 1989) was found to be conserved in *L. major* GP63-6 (position 263-268, Fig. 7). Two *N*-linked glycosylation site were also found to be conserved between *L. major* GP63-6 and *L. major* GP63-1 (position 300 and 407, Fig. 7). Conservation of these features indicates their importance in the structure and function of the protein. Also conserved between the two *L. major* GP63 proteins were regions likely involved in the processing of the translated gene product. These features include the prepeptide leader sequence and the propeptide region proposed to be involved in regulation of proteinase activity via the cysteine switch mechanism (Button *et al.* 1993; Macdonald *et al.* 1994). Comparison of *L. major* GP63-6 to GP63 proteins encoded in other *Leishmania* species indicated that these regions are highly conserved suggesting the importance of these properties in GP63 function and processing.

Despite the similarities between the two *L. major* GP63 predicted proteins, differences were observed. *L. major* GP63-6 was predicted to contain two additional *N*-linked glycosylation sites. The carboxy terminus region encoded by *L. major gp63-6* was also found to be considerably different with an additional 41 amino acid residues in comparison to *L. major* GP63-1. An internal region of differences (positions 421-480, Fig. 7 and 8) was also found between the two proteins. Of particular interest is the point of sequence divergence at the carboxy terminus between the two forms of GP63. The sequence of *L. major* GP63-6 was found to diverge 2 amino acid residues before the asparagine at position 577 (Fig. 7, *Lm (#1)*) that has been identified as the acceptor of the GPI anchor in the *L. major gp63-1* protein (Schneider *et al.* 1992). Comparison of all *Leishmania* GP63 proteins suggested that there are at least two distinct forms of GP63 that can be distinguished by the length and sequence of their respective carboxy termini along with other distinct regions (such as the region between position 421-480, Fig. 7 and Fig. 8). Conservation of the extended carboxy termini form of GP63 in *L. major* (an Old World species) with GP63 sequences predicted in three New World species including *L. mexicana* (C1) GP63 and *L. chagasi* (C1) GP63 and to a lesser degree, *L. guyanensis* (N) GP63 suggests that this form of GP63 likely has an important role in the biology of *Leishmania*. Conservation of GP63 proteins with the shorter carboxy termini (exemplified by GPI-linked *L. major* GP63-1) across different *Leishmania* species also indicates the importance of this form of the protein. The fact that the extended carboxy termini form of GP63 and the shorter GPI-linked form of GP63 are present within the same species suggests that each form may play a different and important role in the life cycle of *Leishmania*.

Results of FACScan analysis of *L. donovani* transfected with pLEXLMGP63-6 suggests that *L. major* GP63-6 is expressed as a surface protein in transfected *L. donovani* promastigotes (see Fig. 13: 3a and b). Hydropathy plot analysis predicted that the carboxy terminus of *L. major* GP63-6 could contain a transmembrane spanning segment since this region contains a hydrophobic stretch of 20 amino acids (position 611-630, Fig. 7). This hydrophobic stretch however could be involved in the cleavage of the carboxy terminus for attachment of a GPI anchor. Predicting GPI addition sequences is difficult due to the lack of a defined consensus

sequence. However, cleavage sites generally have a combination of 2 of the amino acids Ala, Asn, Asp, Gly, or Ser followed by a run of hydrophobic amino acids 10 - 12 residues after the cleavage site (Ferguson and Williams 1988). The *L. major* GP63-6 protein contains several potential cleavage sites upstream of the hydrophobic sequence at positions 611-630 indicating the possibility that this protein may be GPI linked. Western blot results of *L. donovani* transfected with *L. major gp63-6* in this study would support this possibility. As shown in the results section, the 55 kDa protein observed in the CP3.235 western blot analysis of *L. donovani* transfected with pLEXLMGP63-6 (Fig. 14: 1a, lane 3 +) is likely *L. major* GP63-6 since over 99% of the transfected cells were shown to express GP63 detected by the *L. major* GP63 specific mAb #96 in FACScan analysis (see Fig. 13: 3a). Since the protein encoded by *L. major gp63-6* is 42 amino acids longer than the GPI linked protein in *L. donovani* (see Fig. 7) it would be expected to have a higher molecular weight than at least one of the *L. donovani* endogenous bands. (It is reasonable to assume that one of the bands detected in lysates of *L. donovani* control cells [lane 1 (+), Fig. 14: 1a] would represent the endogenous GPI-linked GP63 protein expressed by *L. donovani*). However, since the molecular weight of the GP63 protein in lane 3 (+) (Fig. 14: 1a) is not higher than endogenous *L. donovani* GP63, cleavage at the carboxy terminus of *L. major* GP63-6 and subsequent attachment of a GPI anchor would be consistent with its apparent molecular weight. It has been proposed that both the *L. mexicana* (C1) and *L. chagasi* (C1) forms of GP63 are not GPI linked proteins (Ramamoorthy *et al.* 1992; Medina-Acosta *et al.* 1993).

An attempt to determine whether or not GP63 expressed in the *L. major gp63-6* transfected *L. donovani* promastigotes is GPI linked was made by treating the transfected *L. donovani* promastigotes with phosphatidylinositol phospholipase C (PI-PLC) which cleaves proteins from GPI anchors. FACScan analysis of the positive control cells (*L. donovani* transfected with *L. major gp63-1*) using mAb #96 however failed to detect any removal of *L. major* GP63 protein (data not shown). These negative results may reflect possible steric hindrance of the enzyme by the highly abundant LPG molecules present on the surface of promastigotes. Alternatively, the PI-PLC enzyme may have been limiting since LPG itself is

attached to the membrane by a GPI anchor and occurs in excess of 5 x 10^6 copies per cell (Turco, 1990). Competition for substrate GPI anchors from LPG could have resulted in limitation of the PI-PLC enzyme.

Formal proof of GPI-linkage of *L. major* GP63-6 remains to be determined. Immunoprecipitation with mAb #96 of *L. major* GP63-6 expressed in transfected promastigotes metabolically labeled with [^{3}H] myristic acid or [^{14}C] ethanolamine followed by analysis with PI-PLC would determine whether or not *L. major* GP63-6 is expressed as a GPI-linked protein.

Northern blot analysis of *L. major* RNA from log and stationary phase promastigotes and *in vivo* lesion amastigotes revealed that expression of *L. major gp63-1* transcripts is predominantly found in the promastigote stage whereas transcripts from *L. major gp63-6* are constitutively expressed in both the promastigote and amastigote life stage. These results correlate with expression of different forms of gp63 in the two life stages of *L. mexicana* (Medina-Acosta *et al.* 1993). *L. mexicana (C1)* gp63 encoding the extended carboxy terminus form of GP63 like its *L. major gp63-6* counterpart is expressed in both the amastigote and promastigote life stage. The other two classes of the genes referred to as *L. mexicana C2* and *C3* gp63 were detected only in the promastigote stage which is analogous to *L. major gp63-1* expression observed in this study. If *L. mexicana* (C1) GP63 is not GPI linked as proposed, it is very likely that *(C2)* and/or *(C3)* encode the GPI form of GP63 in *L. mexicana* promastigotes. The GPI-linked form of GP63 has been estimated to represent 90% of the GP63 found in *L. mexicana* promastigotes (Medina-Acosta *et al.* 1989). Northern blot analysis of *(C2)* and *(C3)* expression in *L. mexicana* indicated that *(C2)* and *(C3)* were the predominant gp63 transcripts in promastigotes of this species. In *L. chagasi*, two very similar forms of gp63 which differ in their 3' untranslated regions are expressed separately in the log or stationary phase of growth in promastigotes. *L. chagasi (L1)* gp63 is expressed in logarithmic phase of growth while *L. chagasi (S1)* gp63 is expressed in stationary phase of promastigote growth. Both of these gp63 genes encode proteins similar to the *L. major gp63-1* encoded protein and contain GPI attachment signal sequences similar to *L. major* GP63-1. A third class of gp63 (*L. chagasi (C1)* gp63) was found to be expressed in both log and stationary phases of promastigote growth

(Ramamoorthy *et al.* 1992). This form of the gene encodes the extended carboxy terminus very similar to that of *L. major* GP63-6. Expression of these genes in *L. chagasi* amastigotes however has not been determined.

Results presented in the present study along with other reports (Ramamoorthy *et al.* 1992; Medina-Acosta *et al.* 1993) indicate evolutionary conservation across both Old and New World species of *Leishmania* of at least two different forms of gp63 genes. One form of gp63 (the shorter form known to encode the GPI-linked protein) appears to be developmentally regulated as it is expressed predominantly in the promastigote stage of *L. major* and likely only in the promastigote stage of *L. mexicana*. The other conserved form of gp63 encoding the extended carboxy terminus is constitutively expressed in both promastigotes and amastigotes of *L. major* and *L. mexicana*. Based on these results, one would predict that the *L. chagasi (C1)* gp63 gene encoding the extended carboxy terminus would also be expressed in amastigotes of this species.

Transfection of *L. donovani* with *L. major gp63-6* and use of the *L. major* GP63 specific monoclonal antibody #96 in flow cytometry FACScan analysis indicated *L. major* GP63 expressed by the gene six transfectants is expressed as a surface protein in promastigotes with over 99% of the cells labeled with the mAb #96 (Fig. 13). Results of FACScan analysis of the *L. major gp63-1* transfected *L. donovani* cells demonstrated that more than 50 % of the transfected cells in both clones observed were not labeled with the mAb #96 (Fig. 13: 2a and b). Although care was taken in picking of single colonies arising on drug selection plates to ensure a clonal population, the high proportion of unlabeled cells could represent a nonclonal population of cells with one population expressing *L. major* GP63 encoded by *L. major gp63-1* and the other population not expressing the *L. major* protein. Integration of the hygromycin resistance gene into the *L. donovani* genome by recombination and subsequent loss of episomal elements could also account for the observed distribution. Alternatively, the distribution observed for the gene 1 transfectants could represent a clonal population of cells which are heterogeneous in expression of the plasmid gene. This may be the result of different copy numbers of the episomal elements carrying the gene in different cells.

Localization of *L. major* GP63-6, if it is synthesized in *L. major* amastigotes is an unanswered question. Frommel *et al.* (1990) were able to detect GP63 in *L. major* amastigotes by immunofluorescence and Pimenta *et al.* (1991) using the same antibody in immunogold labeling were also able to show localization of GP63 on the cell surface of amastigotes. Since *L. major gp63-1* is expressed predominantly in the promastigote stage and since restriction map analysis has shown that the five tandemly repeated gp63 genes in *L. major* are highly conserved, it is reasonable to postulate that all five of the repeats are expressed only in the promastigote stage. If this proves to be the case, the GP63 detected in amastigotes of *L. major* would essentially be the result of translation of the sixth gene transcript therefore accounting for the GP63 present in this life stage of the parasite.

Investigation of *L. mexicana* amastigotes has revealed several important aspects of GP63 expression in this species. Although some GP63 was shown to be GPI linked on the surface of *L. mexicana* amastigotes, the majority of the GP63 was determined to lack a GPI anchor with most of the GP63 localized to the flagellar pocket of the amastigote (Medina-Acosta 1989). Similarly, Bahr *et al.* (1993) found that the majority of the GP63 protein from *L. mexicana* amastigotes was soluble which is consistent with an absence of a GPI anchor. However, in their study, GP63 was localized to the lumen of large lysosomes referred to as megasomes characteristic of this species of *Leishmania* amastigotes. The predominant transcript in the amastigote life stage is the *L. mexicana (C1)* transcript that encodes the extended carboxy terminus. It has been hypothesized that the different extended carboxy terminus could contain a different type of signal sequence that may account for its differential localization in the amastigote stage in this species (Medina-Acosta 1993). Whether or not some of the GP63 expressed in *L. major* amastigotes is differentially localized as in *L. mexicana* remains to be determined. The possibility of GPI-linkage of *L. major* GP63-6 would suggest that it would be expressed as a surface protein in amastigotes. On the other hand, the possibility of GPI linkage in one life stage and not in the other life stage could also account for possible differential localization in the different life stages.

To date, only genes encoding the extended carboxy terminus form of GP63 (*L. major gp63-6* and *L. mexicana (C1)* gp63) have been found to be expressed in the amastigote stages of *Leishmania* suggesting that this form of the protein may have functional significance and relevance during this stage of the life cycle. The GPI linked form of GP63 present on the surface of promastigotes has a pH optimum between 7 and 9 for enzymatic activity (Etges *et al.* 1986). This pH optima would be consistent with an enzymatic role in the neutral environment of the insect gut. It is reasonable to postulate that differences in the amino acid sequences of the protein encoded by *L. major gp63-6* may be necessary for optimal enzymatic activity at a lower pH in the acidic environment of the phagolysosome. In agreement with this hypothesis is the finding that the lysosomal GP63-related protein in *L. mexicana* presumably encode by *L. mexicana (C1)* gp63 was found to have a pH optima between pH 5.5-6 (Ilg *et al.* 1993).

The exact role of GP63 in the amastigote life stage remains to be determined. GP63 has been implicated in macrophage uptake of promastigotes via CR3 receptors (Chang and Chang, 1986; Russell and Wilhelm, 1986; Wilson and Hardin, 1988; Russell and Wright, 1988). Possible expression of the GP63 on the surface of amastigotes may likewise play an important role in uptake of amastigotes by other macrophages following rupture of an infected macrophage. Expression of GP63 may also be important for amastigote survival inside the phagolysosome of amastigotes since previous results have shown that GP63 encapsulated liposomes were able to protect liposomes from degradation (Chaudhuri *et al.* 1989). GP63 could also be envisioned to play a protective role for amastigotes in the defense from proteolytic enzymes involved in the blood meal digestion during reinfection of the sandfly vector. Alternatively, GP63 may have a metabolic role in either the sandfly or in the phagolysosome of host macrophage cells (Bouvier *et al.* 1987).

The finding of two heterogeneous gp63 genes in *L. major* that are conserved in other *Leishmania* species (Ramamoorthy *et al.* 1992; Medina-Acosta *et al.* 1993) suggests that both forms of the protein are functionally important in *Leishmania* parasites. Very similar or identical proteins in mammalian cells have been shown to be expressed in either a GPI-linked form, a secreted form, or transmembrane form. Examples of this include the lymphocyte function-

associated antigen 3 (LFA-3) and the neural cell adhesion molecule (N-CAM). In the case of N-CAM, three different forms of the protein are derived from alternative mRNA splicing from a single gene (Murray *et al.* 1984; Owens *et al.* 1987). Two of the splice products encode integral membrane products with different cytoplasmic domains while the third form encodes the GPI-linked form of the protein (Murray *et al.* 1986; Hemperly *et al.* 1986). LFA-3 has also been shown to have two different forms as a result of alternative splicing. One form is known to be GPI-linked while the other form contains a transmembrane segment (Dustin *et al.* 1987). Since the process of cis splicing has not been found to occur in trypanosomatids, heterogeneous genes encoding very similar proteins such as *L. major* GP63-1 and GP63-6 may be a requirement of these organisms to express very similar yet distinct proteins.

As discussed previously, a very interesting finding of the CP3.235 western blot analysis of lysates of *L. donovani* transfected with *L. major gp63-6* is the apparent replacement of endogenous *L. donovani* GP63 with what appears to be *L. major* GP63-6 (see Fig. 14: 1a, lane 3 +). If this protein proves to be *L. major* GP63-6, the question of how the exogenous GP63 could replace the native GP63 arises. Several possible explanations exist. The apparent replacement could indicate some possible form of regulation of GP63 at either a transcriptional level and/or at a translational level. Cells may have a limit for the amount of GP63 protein which they will express. A protease may target GP63 for degradation to keep levels of GP63 from exceeding maximal levels or alternatively GP63 itself may inhibit its own transcription and/or translation when levels of expression of the protein exceed a certain level. Introduction of excess plasmid DNA into cells by electroporation may have resulted in a high proportion of *L. major* gp63 genes relative to the endogenous *L. donovani* genes. This could result in a high proportion of *L. major* gp63 transcripts relative to *L. donovani* transcripts. If GP63 protein expression is limited to a maximal amount, the majority of the translated products would be from the *L. major* transcripts and therefore a low level of endogenous translated products may not be detected in western analysis. Alternatively, *L. major* GP63-6 may be more stable than endogenous *L. donovani* GP63. Another possible explanation although less likely is the possibility of chromosomal integration of *L. major gp63-6* by homologous recombination and possible replacement of *L.*

donovani gp63 genes. Assuming all *L. donovani* gp63 genes are transcribed, replacement of 24 genes (12 genes per chromosome) would have to occur for complete replacement of the endogenous gp63 genes making this explanation unlikely. It has also been shown that circular plasmid DNA is maintained in transfected *Leishmania* cells as circular extrachromosomal elements with little or no chromosomal integration (Webb et al. 1994; Kapler *et al.* 1990; Laban *et al.* 1990; LeBowitz *et al.* 1990) further suggesting that replacement of endogenous *L. donovani* gp63 genes with *L. major* gp63 genes by homologous recombination is unlikely.

Formal proof that GP63 detected in FACScan and western blot analysis of *L. donovani* pLEXLMGP63-6 transfectants is encoded by the *L. major gp63-6* remains to be determined. One cannot rule out the possibility that the GP63 detected in the gene six transfectants could be the result of a recombination event whereby a 5' portion of the *L. major gp63-6* gene is recombined with the 3' portion of the endogenous *L. donovani* gp63 gene containing the GPI attachment signal sequence. This event would result in a gene which could encode a chimeric GP63 protein containing an *L. major* region recognized by the mAb #96 and the GPI attachment carboxy terminus encoded by the endogenous *L. donovani* gp63 gene. Again, this explanation seems highly unlikely since recombination would likely have to occur with all endogenous *L. donovani* gp63 genes and since it has been shown that recombination involving circular plasmid DNA is a rare event (Webb et al. 1994; Kapler *et al.* 1990; Laban *et al.* 1990; LeBowitz *et al.* 1990).

Another interesting finding of this study is the observation of developmental regulation of *L. major gp63-1* and the constitutive expression of *L. major gp63-6* which is similar to expression of similar gp63 genes in other species (Medina-Acosta *et al.* 1993; Ramamoorthy *et al.* 1992). Developmental regulation of *L. major* gp63 genes could occur at the level of transcription and/or mRNA stability. Current evidence suggests that an important mode of regulation in Kinetoplastids is mRNA stability. Investigation into the expression of the three different classes of gp63 genes in *L. chagasi* promastigotes has shown that mRNA stability plays an important role in the expression of some forms of gp63 transcripts (Wilson *et al.* 1993). The expression of the log phase *L. chagasi (L1)* gp63 transcripts was found to increase dramatically in the presence of cycloheximide, an inhibitor of protein synthesis. It was proposed that treatment with

cycloheximide prevented the synthesis of a negative regulatory protein which specifically targets log phase transcripts for degradation resulting in the increase of log phase transcripts. Since the 5' region of all forms of gp63 transcripts are essentially the same, regions in the 3' untranslated region (UTR) were proposed to be involved in targeting of the negative regulator (Wilson *et al.* 1993). Interestingly, over 580 base pairs of the 3' flanking region of *L. major gp63-6* were shown to be similar to 570 base pairs of the 3' UTR of the *L. chagasi (C1)* gp63 3' as well as to the 48 base pairs of the *L. mexicana (C1)* gp63 3' UTR reported (see Fig 5). The similarity of the *L. major gp63-6* and *L. mexicana (C1)* gp63 3' UTR sequences suggests that this 3' region may be relevant in expression of these transcripts in both life stages in these two species. This hypothesis would predict that the *L. chagasi (C1)* gp63 would be expressed in the amastigote stage of this species since its' 3' UTR is very similar to the 3' flanking region of *L. major gp63-6*. The *L. major gp63-1* gene transcript may contain sequences relevant to targeting by a negative regulator as in the case of *L. chagasi (L1)* gp63. However, comparison of the 3' flanking region of *L. major* did not show any identity with any of the 3' UTRs reported for any other *Leishmania* gp63 genes (data not shown). Nuclear run on assays would determine whether regulation of gp63 genes in *L. major* promastigotes and amastigotes is at a transcriptional level or at a post-transcriptional level involving the possibility of mRNA stability.

CONCLUSIONS

1. GP63 is encoded by a heterogeneous gene family in *L. major*. *L. major gp63-1* encodes a GPI-linked protein while *L. major gp63-6* encodes a very similar form of GP63 but with a different and extended carboxy terminus. Whether or not the *L. major* GP63-6 is GPI linked remains to be determined.

2. Northern blot analysis demonstrated that *L. major gp63-1* is expressed predominantly in the promastigote stage while *L. major gp63-6* is expressed constitutively in both the promastigote and amastigote stage.

3. The *L. major* anti-GP63 mAb #96 recognizes surface expressed GP63 in *L. donovani* promastigotes transfected with *L. major gp63-6* suggesting that *L. major* GP63-6 is expressed as a surface protein.

4. Expression of *L. major gp63-6* in amastigotes suggests that *L. major* GP63-6 likely plays an important role in the amastigote stage of *L. major*.

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