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Leishmania chagasi surface glycoprotein GP46 functions in parasite resistance to lysis by complement

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CHAPTER 1: LITERATURE REVIEW

Leishmania spp. are parasitic protozoa that infect an estimated 1.5 to 2 million people annually in 88 countries. Currently no effective vaccines are available, and although treatments exist emergence of drug resistant strains is a major concern. *Leishmania* spp. cause a range of diseases varying in severity from the self-healing skin lesions of the cutaneous form to the potentially fatal visceral form. The cutaneous form is considered a childhood illness in endemic areas, while the visceral form infects 500,000 people annually.

Leishmania has a digenetic life cycle, which alternates between a mammalian host and a sandfly vector. The parasites are introduced to the bloodstream of the mammal by the bite of an infected sandfly. In the mammal, the parasites are called amastigotes and live and reproduce inside the phagolysosome of macrophages. As the cells divide and multiply in the macrophage, they eventually cause the macrophage to burst, releasing free amastigotes that can then infect more macrophages. When a sandfly then takes a bloodmeal from an infected mammal, the ingested parasites infect the sandfly.

Once in the sandfly, the parasites change from the amastigote to the promastigote form. These promastigotes must then escape the chitinous peritrophic matrix that surrounds the bloodmeal. Once outside of the peritrophic matrix, the promastigotes travel up the midgut to the cardiac valve. Normally, the cardiac valve is responsible for allowing the unidirectional flow of blood during a bloodmeal. In infected sandflies flow is bidirectional due to damage to the cardiac valve caused by the parasites and parasite accumulation. This allows the infective promastigotes to be regurgitated into the mammal when an infected sandfly takes a bloodmeal (reviewed in Schlein, 1993).

During the 1-3 weeks in the fly gut, or in laboratory cultures in the absence of sandflies (Sacks and Perkins, 1984), parasites undergo a process termed metacyclogenesis. Metacyclogenesis is the process by which the promastigotes differentiate from a non-

infectious procyclic form to a highly infectious metacyclic form. Notable changes that occur in metacyclogenesis are modifications to infectivity, morphology, replication state, and surface biochemistry. The procyclic form replicates by binary fission, is rounded with a short flagellum, and has low infectivity to mammals. The metacyclic form does not replicate, is highly motile with an elongated shape and a long flagellum and is highly infectious to mammals. The biochemical changes on the surface include an increase of surface glycoprotein GP46 in the metacyclic forms, as well as a change in the structure of surface lipophosphoglycan (Beetham et al. 1997, Sacks et al. 1992 McConville et al. 1990). The surface glycoprotein GP63 has three forms, one is expressed constitutively, one only in procyclic promastigotes, and the other only in metacyclic promastigotes (Roberts et al., 1995).

Although many proteins, including the surface proteins noted above, have been characterized to be differentially expressed during parasite development, and although these genes are transcribed by RNA polymerase II (i.e. α-amanitin sensitive), no promoters for RNA polymerase II have been found in *Leishmania* spp. All differentially expressed genes characterized thus far are constitutively transcribed on polycistronic mRNAs. These polycistronic mRNAs are trans-spliced with a splice leader RNA, which adds an identical splice leader RNA sequence to the 5' end of all mature mRNAs. Poly-adenylation of the mRNAs is coupled with the addition of the splice leader to the downstream gene, as shown in experiments where shortening or lengthening of the intergenic region between the poly-adenylation site of one gene and the splice leader site of the downstream gene results in a shift of the poly-adenylation site of the upstream gene corresponding to the insertion or deletion (reviewed in Ullu and Nilsen, 1995).

GP46, GP63 and a heat shock protein (HSP83) are among the best characterized of developmentally regulated genes in *Leishmania*. For all of these genes, modulation of the steady state levels of the mRNAs requires RNA elements contained within their 3'

untranslated region (Beetham et al. 1997, Myung et al, 2002, Shapira et al., 2001, Ramamoorthy et al., 1992). Interestingly, the recent sequencing of chromosome 1 *in L. major* showed that all predicted open reading frames were arranged in the same direction starting near the center of the chromosome, suggesting there may be as few as two transcription start sites on this 1 Mb chromosome (Myler et al. 1999).

The lipophosphoglycans (LPGs) are the most abundant molecule on the surface of the parasites. The LPGs have four domains, which are a phosphatidylinositol anchor, a glycan core, disaccharide phosphate repeat units, and a small oligosaccharide cap. The LPG of *L. donovani* has been shown to double the length of the repeats in metacyclic promastigotes (Sacks et al. 1995). It has also been shown that in *Leishmania major* the LPGs of metacyclic promastigotes have galactose side chains that are capped with arabinose, while in procyclics the terminal sugars are galactose (McConville et al. 1992).

GP63 (also called MSP) is a surface glycoprotein that is the second most abundant surface molecule and has three forms based on patterns of expression. One form is expressed constitutively, one only in logarithmically growing promastigotes, and the other only in stationary phase promastigotes. GP63 has been shown to be a serine protease with a wide range of substrates, including serum albumin, hemoglobin, C3, immunoglobulin G and rat liver lysosomal proteins (Chaudhuri and Chang, 1988). Most importantly to this work, it has been determined that GP63 degrades C3 into a form antigenically similar to iC3b (Brittingham et al. 1995), which is an inactive form of C3 that prevents the complement cascade from continuing through to the formation of the membrane attack complex but can promote the phagocytosis of the parasite by macrophages.

GP46 (also called M-2 or PSA) is a surface glycoprotein that is present in all *Leishmania* species studied so far except for members of the *L. braziliensis* complex (McMahon-Pratt et al. 1992) and is a multicopy gene that accounts for approximately 1-2% of the total membrane protein in metacyclic cells (Kahl and McMahon-Pratt 1987). All

GP46 sequences determined so far have a serine/threonine rich region, 3-13 leucine-rich repeats of 24-25 amino acid residues, a hydrophobic amino-terminal that signals for translation on the rough endoplasmic reticulum, and a carboxyl-terminal which is most likely cleaved when the protein is attached to a glycolipid anchor (Jimenez-Ruiz et al. 1998). Although leucine rich repeat regions are believed to play a role in protein-protein interactions, GP46 is not homologous to any protein with a known function. Immunization with GP46 has been shown to be protective against *Leishmania amazonensis* infection in rodent models of disease (Champsi and McMahon-Pratt, 1988; McMahon-Pratt et al. 1993), but until now a function for this protein has not been determined.

GP46 is encoded by a gene family containing multiple non-identical copies of GP46 genes that are arranged in clusters (McMahon-Pratt et al. 1992; Beetham et al. 1997). Northern blot analyses in *Leishmania chagasi* have demonstrated two bands of 2.8 kb and 4.8 kb that increase over 30-fold in abundance as parasites change from procyclic to infective metacyclic promastigotes. It is believed these are the products of two classes of GP46 genes, *gp46A* and *gp46B* (Beetham et al., 1997). Western blot analysis with total lysates of *L. chagasi* show a similar increase in two bands of 44 and 60 kDa (unpublished data and Chapter 2).

In the lab, *Leishmania* spp. are maintained by a series of alternate passages through hamsters and culture. In culture, growth and development mimics what is observed in the sandfly vector in terms of replication, infectivity and morphology (Sacks and Perkins, 1984). However, without passage through a hamster, parasites become attenuated (Howard et al. 1987, Nolan and Herman, 1985, De and Roy, 1999). These high passage parasites are much less capable of causing infections in rodents or in isolated macrophages, expression of GP63 is perturbed (McGwire and Chang, 1994), and expression of GP46 ceases (unpublished data, Chapter 2). High passage cells still undergo the morphological change from round to long and slender promastigotes.

One of the first host anti-microbial immune mechanisms the promastigotes encounter when inoculated into a mammalian host is complement mediated lysis (CML). Three different pathways can activate complement: classical, mannose-lectin binding, and alternative. All three pathways can have the same end result of the formation of a membrane attack complex, which is a ring of proteins that punctures the membrane, forming pores and killing the cells. The alternative pathway is activated by the parasite surface and does not rely on recognition of the parasite by antibodies, making it an innate immune response. The classical pathway is dependent on antibody recognition.

It is well documented that many *Leishmania* species activate complement via the alternative pathway, although a few species have been shown to also activate the classical pathway (Mosser and Edelson 1984, Mosser et. al. 1986, Dominguez 2001). While procyclic promastigotes of all *Leishmania* spp. are sensitive to CML, metacyclic promastigotes are differentially sensitive to CML. It has been demonstrated that *L. panamensis* and *L. donovani* metacyclic promastigotes are resistant to CML while *L. major* metacyclic promastigotes are susceptible to CML (Franke et al. 1985).

In *Leishmania* spp., roles for GP63 and LPG in resistance to complement mediated lysis have both been studied. As stated earlier, GP63 has been shown to cleave C3 into a form antigenically similar to iC3b (Brittingham et al., 1995). In this same study it was determined that *L. amazonensis* deficient in GP63 expression or that expressed a proteolytically inactive form of the enzyme were susceptible to lysis by normal guinea pig serum. The parasite line deficient in GP63 expression was transfected with an expression vector containing the GP63 open reading frame, and these transfectants showed a significant increase in survival in 20% normal guinea pig serum. It was also shown that GP63, both native and proteolytically inactive, promoted opsonic complement fixation and binding to the human complement receptors CR1 and Mac-1 which may facilitate invasion of macrophages.

However, GP63 is not solely responsible for complement activation by these parasites (Brittingham et al., 1995).

The LPGs of *L. major* are also believed to play a role in resistance to complement mediated lysis. The LPG of both log and stationary *L. major* are the main C3 acceptor of the parasites (Puentes et al., 1988). In both log and stationary cultures a membrane attack complex forms, but in stationary phase cells the complex is shed from the surface of the parasite and does not cause lysis (Puentes et al., 1990). It is believed that this shedding is due to the elongation of disaccharide repeats in the LPG in stationary phase, and this is similar to a mechanism of complement resistance in Salmonella that is due to long O-polysaccharide side chains on lipopolysaccharide molecules (Joiner et al., 1982).

Due to the abundance of GP46 on the surface of the infective promastigote membrane, the conservation of GP46 among *Leishmania* spp., and the roles of other *Leishmania* surface proteins in resistance to CML, we hypothesized that expression of GP46 plays a role in resistance to CML. We tested this hypothesis by re-expression of GP46 in *L. chagasi* promastigotes that had been serially passed in culture and testing their resistance to complement mediated lysis using normal human serum.

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CHAPTER 2. *LEISHMANIA CHAGASI* SURFACE GLYCOPROTEIN GP46 FUNCTIONS IN PARASITE RESISTANCE TO LYSIS BY COMPLEMENT.

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Abstract

The mRNA for GP46, an abundant surface glycoprotein of most *Leishmania* spp., is expressed at high levels only in infectious *Leishmania chagasi* promastigotes. To investigate a possible role for GP46 in infectivity we analyzed the relationship between GP46 and resistance to complement-mediated lysis (CML). *L. chagasi* promastigotes passed for 2-3 weeks in culture were about 9-times more resistant to CML (in the presence of 25 and 50% human serum) when in stationary phase of growth than in logarithmic phase of growth. Cells passed for >100 weeks were extremely sensitive to CML regardless of the phase of growth. GP46 expression levels paralleled CML resistance phenotype: low to non-detectable in higher passage cells (cells passed for 15-18 weeks, or for >100 weeks) and in low passage cells in logarithmic growth phase. Earlier studies with *L. chagasi* reported the identification of GP46A and GP46B coding sequences, and these varied mainly in the number of leucine rich repeats found in the amino-terminal half of the predicted protein sequences. GP46A and GP46B were re-expressed in higher passage cells transformed with pXβGAL2 plasmids that contained GP46 coding sequence in place of β-galactosidase coding sequence. These higher passage cells were significantly more resistant to CML ($p<0.01$ when incubated with 25 and 50% serum) as compared to control cells transfected with the unmodified pXβGAL2 plasmid: at 50% serum, about 80% versus 20% survival in the controls. Levels of CML resistance in the GP46-expressing higher passage cells approached that seen in low passage stationary phase cells. The data support a role for GP46 in evasion of lysis by complement.

Introduction

Leishmania spp. are protozoan parasites that infect a variety of vertebrates, including 1.5-2 million people annually. These parasites cause a wide range of diseases, collectively called leishmaniases, that present as self-healing skin lesions (cutaneous leishmaniasis), destroyed mucous membranes (mucocutaneous leishmaniasis), and potentially fatal involvement of internal organs including the spleen and liver (visceral leishmaniasis). *Leishmania* spp. are digenetic, alternating between an amastigote form in the mammalian host and a promastigote form in the sandfly vector.

Through a process called metacyclogenesis, promastigotes undergo a series of developmental changes while in the sandfly vector that result in the formation of highly infectious metacyclic promastigotes (Schlein, 1993). Metacyclogenesis results in changed morphology, replicative state, infectivity to mammals and surface biochemistry. These changes are also observed *in vitro* as parasites progress from logarithmic (non-infectious to mammals) to stationary (highly infectious to mammals) phase of growth in liquid culture (Sacks and Perkins, 1984). Among the surface biochemistry changes associated with metacyclogenesis are an increased length of phosphoglycan repeats in lipophosphoglycan (LPG) (Sacks et al., 1995), the expression of different forms of the surface protease GP63 (Roberts et al., 1995), and increased expression of surface glycoprotein GP46 (Beetham et al., 1997).

When metacyclic promastigotes are passed into the bloodstream of a mammal upon bite of an infected sandfly, one of the first immune mechanisms encountered is complement-mediated lysis (CML). All *Leishmania* species studied are sensitive to CML in the logarithmic phase of growth, but sensitivity is variable when in the stationary growth phase. For example, stationary growth phase *Leishmania panamensis* and *Leishmania donovani* are resistant to CML, but *Leishmania major* is not (Franke et al., 1985).

Studies have shown the potential importance of two abundant surface molecules, LPG and GP63 (also known as major surface protease or MSP) to promastigote resistance to CML (Brittingham et al., 1995; Puentes et al., 1990). The lengthening of LPG is hypothesized to function in resistance to CML by causing shedding of the membrane attack complex, the complex by which CML is effected, from the parasite surface (Puentes et al., 1990). GP63 is thought to function by proteolytically cleaving complement factor C3b to a form that is unable to support formation of the membrane attack complex (Brittingham et al., 1995).

Although, as noted above, growth of promastigotes in culture is similar to growth in the fly, studies have shown that repeated passage in culture attenuates parasite infectivity to cultured macrophages and animals (Howard et al., 1987; De and Roy, 1999; Nolan and Herman, 1985). Molecular events that are associated with this attenuation are an increase in galactosyl transferase activity in high passage promastigotes (De and Roy, 1999), and an increased sensitivity to CML (Howard et al., 1987).

GP46 (also known as parasite surface antigen or PSA), like GP63 and LPG, is an abundant surface macromolecule, and accounts for 1-2% of the total membrane protein in metacyclic *Leishmania amazonensis* promastigotes (Kahl and McMahon-Pratt, 1987). GP46 is found in all *Leishmania* spp. examined except members of the *Leishmania braziliensis* complex (McMahon-Pratt et al., 1992). Where characterized, GP46 is encoded by multiple non-identical genes (McMahon-Pratt et al., 1992, Beetham et al., 1997). In *Leishmania chagasi*, the steady state level of GP46 mRNA increases over 30-fold as the parasites grow to stationary phase (Beetham et al., 1997), but GP46 mRNA expression is reduced to undetectable levels after repeated passage of promastigotes in culture. A function for GP46 has not been previously determined, although the protein does contain leucine-rich repeats of 24-25 residues which have been shown in other proteins to function in protein-protein interactions (Jimenez-Ruiz et al., 1998). Because GP46 mRNA abundance in *L. chagasi* is

high only in infectious promastigotes we hypothesized a function for GP46 in resistance to complement mediated lysis.

Materials and Methods

Maintenance of *Leishmania* cultures

L. chagasi promastigotes were maintained as described in culture at 25˚C in a modified minimum essential media (HOMEM) supplemented with hemin and heat-inactivated fetal calf serum (Ramamoorthy et al., 1992). Promastigotes were seeded at a 1 X 10^6 cells/ml and harvested by centrifugation in logarithmic or stationary phase of growth, which was determined by morphology and cell concentration (Zarley et al., 1991). For the purpose of experiments reported here, logarithmic phase cells were harvested when culture concentrations were 0.75-1.0 X 10^7 cells/ml, which occurred 2.5-3.5 days after seeding the culture. Stationary phase cells were collected after seven days in culture, which was two days after cells reached maximum density. HOMEM used for cultures of transformed promastigotes was supplemented with 50μg/ml Geneticin® (Invitrogen, Carlsbad, CA).

Construction of Plasmids

The isolation and sequencing of *L. chagasi* cDNA for GP46A and a lambda-phage genomic clone of GP46B was described previously (Beetham et al., 1997). The first six residues of the deduced proteins are identical: MALCVR. Expression vector pXβGAL2, kindly provided by Stephen Beverley (LeBowitz et al., 1990), was digested with *Bam* HI followed by mung bean nuclease treatment to blunt end the DNA to eliminate the entire β-galactosidase coding sequence except for the first six nucleotides that code for MA amino acid residues. Subsequent digestion with *Xba* I, which cleaves 3' to the original β-gal insert, yielded a pXβGAL derivative vector suitable for directional cloning. The coding sequence of GP46A and B were amplified by PCR using forward primer

5'gcatacgtactgtgcgtgcgtcggctggt3' (GP46A and B) and reverse primers 5'ggttctagatcacgccgccagccccacgc3' (GP46A) and 5' gctactagttcacgtcgccagccccacg3' (GP46B). The forward primer contains a *Sna* BI restriction endonuclease site (*Sna* BI produces blunt-ended products), while the reverse primers contain sites that allow ligation to *Xba I*-digested sites (the GP46A and B primers include *Xba* I and *Spe* I sites respectively). Following PCR amplification (35 cycles of 94°C 1 min., 58°C 1 min., 72°C 1.5 min.), PCR products were digested with *Sna* BI and *Xba* I (GP46A) or *Spe* I (GP46B), band isolated on low gelling temperature agarose, and ligated into pXβGAL2-derived vector previously prepared as described above. Due to the *Sna* BI 1/2 site, the resulting constructs code for GP46A and B that contain a new Valine residue at position three (MAVLCVR). Inserts were verified by sequencing (University of Iowa and Iowa State University DNA and Sequencing Facilities using Applied Biosystems Model 377 Prism DNA sequencers).

Generation of transfectants

L. chagasi promastigotes that had been in culture for less than three weeks after initiating the culture using hamster-derived amastigotes were stably transfected by electorporation with pXβGAL2, pXGP46A, or pXGP46B. Clones were isolated on semi-solid medium supplemented with 50 µg/ml Geneticin® (Invitrogen, Carlsbad, CA) according to published protocol (LeBowitz, 1994). Clonal isolates were then serially passed.

PCR Confirmation of transfectants

Transfectants containing the pXGP46A or pXGP46B plasmids were confirmed using PCR. Plasmids were isolated from transfected parasites using the alkaline lysis miniprep method (Sambrook and Russell, 2001). PCR reactions used 1µl of DNA isolated from transfectants as the template and other components as specified in the protocol for Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA). Reactions consisted of 30 cycles of 94°C

1 minute, 50°C 1 minute, and 72°C 2 minutes. The DNA used as positive controls were pXGP46A and pXGP46B. The forward primer for GP46A and B analysis was 5'-gtggtctgcgatgccga-3'. The reverse primer for all reactions, designed using the sequence located 3' to the GP46 insert sites in the plasmid vector, was 5'-caccccaggctttacac-3'. Negative control reactions excluded template DNA, the forward primer, or the reverse primer from the reaction. Products were analyzed by agarose gel electrophoresis.

Preparation of human serum

Human blood was drawn from naïve, adult humans in serum tubes and incubated at room temperature for 45 minutes, then on ice for 45 minutes. Coagulated blood was centrifuged for 15 minutes at 2000Xg at 4°C. Serum was then allocated into eppendorf tubes for single use and stored at -80°C until use. If necessary, serum was heat inactivated by incubation at 56°C for 15 minutes.

Complement Lysis Assay

Promastigotes were incubated at 37°C for 30 minutes in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4) containing 0%, 12%, 25%, or 50% normal human serum with a final cell density of 3.5 X 10^7 cells/ml. After incubation, samples were immediately diluted 100-fold with ice-cold PBS and intact, motile promastigotes counted by light microscopy on a hemocytometer. Results are expressed as the percentage of live promastigotes as compared to control reactions that contained no serum. For statistical analysis, means from the pXGP46A and pXGP46B transfectants were compared to pXβGAL2 clones using a one-way ANOVA followed by a Dunnett's test using pXβGAL2 as the control.

Western Analysis

Total lysates of parasites were separated by SDS-polyacrylamide gel electrophoresis (Laemlli, 1970) using a 10% separating gel, then electro-transferred to nitrocellulose membranes using a semi-dry transfer apparatus at 2 mA/cm^2 for 1 hour in Towbin buffer (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3). Blots were incubated at 25°C (i) for 1 hour in PBS-T (PBS plus 0.05% Tween-20) supplemented with 5% powdered milk, then (ii) for 1 hour in PBS-T supplemented with either primary rabbit antisera to GP46 diluted 1:2000 or a monoclonal antibody to tubulin diluted 1:5000. After washing the blots in PBS-T 4 times for 15 minutes per wash, appropriate secondary HRP-conjugated (anti-rabbit or anti-mouse) antibody (Pierce, Rockford, IL) was added at a 1:30,000 dilution in PBS-T and incubated for 1 hour at 25°C. Blots were then washed 4 times as above, and antibody binding was visualized by enzyme linked chemiluminescence per instructions (SuperSignal ®, Pierce, Rockford, IL). Blots initially probed with antisera to GP46 were stripped by incubation at 37°C for 30 minutes in 1M glycine to be reprobed with the tubulin monoclonal antibody.

Anti-GP46 antisera was isolated from rabbits immunized with a recombinant GP46A-Glutathione S-transferase (GST) protein conjugate produced in *Escherichia coli* using the pGEX4-T-1 plasmid system (Amersham Pharmacia Biotech, Piscataway, NY). A cDNA clone containing a GP46A 3'-untranslated region plus all but the first 112 bases of the GP46A coding sequence was previously described (Beetham et al., 1997). The insert of that clone was bordered by *Eco* RI (5' end) and *Xho* I (3' end) restriction endonuclease sites, which allowed its cloning, in-frame, into the same restriction sites of pGEX4T1. Standard procedures were used to purify the recombinant protein antigen from *E. coli* lysate (Amersham Pharmacia Biotech, Piscataway, NY) by binding to, followed by elution from, a glutathione-based column, then separation by preparative SDS-PAGE.

The tubulin-specific monoclonal antibody was developed by M. Kymkowsky and was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Results

Expression of GP46 in high passage *L. chagasi* promastigotes

Earlier studies showed that GP46 mRNA levels in low passage *L. chagasi* promastigotes increase about 30-fold in cells at stationary phase of growth (Beetham et al., 1997), and this increased mRNA abundance correlates with increased abundance of the protein (data not shown). When cells were serially passed 15 times, GP46 levels in stationary phase promastigotes declined by 85% (Fig. 1, lane 3 versus 1). Cells passed weekly for more than 2 years in culture had no detectable GP46 when analyzed using the same analytical procedures used here (data not shown).

Expression plasmids were made by replacing the coding sequence of β-galactosidase with that of either GP46A or GP46B in the expression construct pXβGAL2, and constructs were confirmed by sequencing the entire inserts and by restriction endonuclease digestion. *L. chagasi* promastigotes were transfected within their first three passages in culture, then clonal isolates were carried through to higher passage under drug selection. Presence of the desired plasmid was confirmed by PCR (Figure 2). In this analysis, PCR amplification with a single forward primer specific to GP46A and B, and a reverse primer specific to a region of the vector downstream of the open reading frame, yielded products of the expected size (710 bp for pXGP46A and 860 bp for pXGP46B plasmids). The 150 bp difference in length of these products is due to the longer coding sequence of GP46B. PCR of positive controls yielded the expected 710 and 860 bp products (Fig. 2) that were not present in negative controls (data not shown.

Western analysis of transfectants using antisera raised against GP46 demonstrated that relatively high levels of GP46 were produced in cells passed 15 times that contained the

plasmids bearing the GP46A or GP46 B coding sequences (Fig. 1, lanes 4 and 5, the 44 and 60 kDa bands). By comparison, GP46 was almost non-detectable in cells at the same passage that contained the control plasmid that bears the coding sequence for β-galactosidase instead of GP46 (Fig. 1, lane 3). On these blots a minor protein migrating at about 54 kDa was detected with the GP46 antisera, but levels of this protein did not increase in cells transfected with plasmids bearing GP46 coding sequence.

To estimate the relative amount of GP46 present in the cells, GP46 signals were normalized to that of tubulin. GP46 levels were determined by western blot using anti GP46 antisera (Fig. 1, upper panel). Bound GP46 antibodies were stripped, then tubulin levels were determined by reprobing the blot using a monoclonal antibody to tubulin (Fig. 1, lower panel). Densitometric analysis of the autoradiographs resulting form these experiments indicated the tubulin signal varied less than 35% among all lanes. After using tubulin signals to normalize GP46 signals for between-lane loading differences, GP46 levels in passage 15 cells transfected with pXGP46A and pXGP46B were seen to be 1.5- and 3.7-times that of low passage cells. The analysis also indicated that GP46 levels in cells transfected with pXGP46A and pXGP46B were 9- and 24-times that of cells transfected with control (pXβGAL2) plasmid (Fig. 1, lanes 4 and 5 compared to 3).

Resistance of parasites to complement mediated lysis

L. chagasi low passage promastigotes are very resistant to CML when in stationary, but not logarithmic, growth phase (Fig. 3a). Stationary phase promastigotes were approximately 9-times more resistant to CML at 25% and 50% serum than were logarithmic phase promastigotes. Promastigotes passed over 100 times were highly susceptible to CML when in either logarithmic or stationary growth phases (Fig. 3b). At 12 percent serum, serially passed cultures were more sensitive to CML than were low passage cells (Fig. 3a versus b).

Expression of GP46 in higher passage cells confers resistance to CML (Fig. 3c). Stationary phase cultures of transfected *L. chagasi* promastigotes (including the transfectants for which GP46 levels are shown in Fig. 1) were assayed for sensitivity to CML after 15-18 passages (Fig. 3c). Four clonal isolates of each transfectant were assayed in increasing concentrations of normal human serum. In the presence of 25% and 50% serum, transfectants that expressed plasmid-encoded GP46 were significantly more resistant to CML than were transfectants transfected with control plasmid ($p < 0.01$). Approximately 77% of GP46-expressing cells survived treatment with 50% serum, while only about 22% of the cells transfected with pXβGAL2 survived the same treatment. Thus increased resistance to CML corresponds to expression of GP46 in the transfectants (Fig. 1). While at 25% and 50% serum the resistance of control transformants at passage 15-18 to CML was as low as that of non-transfected cells that had been passed >100 times, the passage 15-18 cells had greater standard deviations. When incubated in 12% serum, no significant difference in resistance to CML was seen among the transfectants ($p > 0.10$). Use of heat-inactivated serum in experiments with transformed or non-transformed cells resulted in 100% survival at all serum concentrations (data not shown).

Discussion

This study was designed to test a hypothesized function for GP46 in resistance to CML. One initial observation, that low passage *L. chagasi* promastigotes in stationary culture phase become highly resistant to CML (Figure 3a), is similar to previous observations of *L. panamensis* and *L. donovani* promastigotes (Franke et al., 1985). Additional characterization showed that serial passage leads to loss of CML resistance (Fig. 3b) and GP46 expression (Fig. 1). Building on these observations, we tested our hypothesis by re-expressing GP46 in higher passage cells then measuring the sensitivity of the cells to CML. This approach is similar to that used by Brittingham et al. in which GP63 was re-expressed in

high passage *L. amazonensis* using the same expression vector used in this study (Brittingham et al., 1995).

The data demonstrate that higher passage cells that re-express GP46 resist CML (Fig. 3c). However, resistance to CML was not quite restored to the level seen in low passage promastigotes. The most likely reason for this is that resistance to CML is a combinatorial process that involves multiple surface macromolecules that, like GP46, are affected by serial passage. Other surface-associated macromolecules thought to be important to CML resistance are LPG and GP63 (Brittingham et al., 1995, Puentes et al., 1990). We and others have observed that serially passed *L. chagasi* and *L. amazonensis* promastigotes have reduced GP63 expression, (Roberts et al., 1995; Liu and Chang, 1992), and serially passed *L. donovani* have increased galactosyl transferase activity and terminally exposed galactose residues (De and Roy, 1999).

The data presented here, in combination with the earlier studies of GP63 and LPG functions, supports a model in which resistance of *L. chagasi* promastigotes to CML may be achieved by several different mechanisms that may work additively or synergistically. An important question is whether the capacity *in vivo* for GP46 to confer CML resistance translates to a biological function of GP46. Although experiments represented here relied on the approach of gene amplification via an episomal expression system, and alternative approach of gene deletion would be another way to validate the biological relevance to our posited GP46 function. For example, if GP46 expression could be abrogated in low passage cells, then studies could determine the corresponding effects on infectivity. Unfortunately, because GP46 is a multi-copy gene where examined (McMahon-Pratt et al., 1992; Beetham et al., 1997), use of gene knockout strategies would be technically very challenging, although this approach was used to remove a 20kb genomic region containing seven closely clustered *msps* genes in *L. major* (Joshi et al, MBP, 2002). RNA interference is an alternative approach towards reducing levels of an mRNA. Unfortunately, this technology that works so

well in *Trypanosoma* spp. has not been made to work in *Leishmania* spp., even when attempted by researchers who routinely perform RNA interference experiments with *Trypanosoma* spp. (unpublished, J. E. Donelson). Gene deletion experiments using inhibitory RNA techniques will have to wait on perfection of this technology in *Leishmania* spp.

It is interesting to note that work by Franke et al. demonstrated that at all serum concentrations >10%, *L. panamensis* stationary promastigotes exhibited greater CML resistance than the other species examined, including *L. donovani* (Franke et al., 1985). Furthermore, *L. panamensis* is a member of the *L. braziliensis* complex, and studies have also indicated that this is the only group of *Leishmania* spp. examined in which GP46 genes or protein have not been detected (McMahon-Pratt et al., 1992). This suggests that in the *L. braziliensis* complex CML resistance due to non-GP46 macromolecules may be high enough to have significantly reduced the evolutionary selective pressure that in other complexes drove for retention of GP46. Of course, there are alternative hypotheses to explain a loss of GP46 in this complex. For instance, since members of this complex develop in the hind-gut of the fly vector, while members of other complexes do not, perhaps GP46 is not required for parasite development in the fly hind gut but is required for development elsewhere in the fly. It is also possible that failure to detect GP46 in the *L. braziliensis* complex was due to factors other than gene absence. At the time the study was done, regulated abundance of GP46 in developing promastigotes was an unknown phenomenon. Perhaps DNA and protein samples from *L. braziliensis* complex members were taken from cells at a non-GP46-expressing growth phase. It is also possible that GP46 genes have diverged sufficiently in the *L. braziliensis* complex to impair their detection with probes made to GP46 of *L. amazonensis*, as were used in the early study. However, this would be a difficult hypothesis to support, given that the same probes used to detect GP46 in a variety of *Leishmania* spp. also hybridized to chromosomes of *Crithidia fasciculata*, a member of the same family

(Trypanosomatidae) as *Leishmania* spp. but that diverged prior to when the *L. braziliensis* complex became evolutionarily isolated from other Leishmania complexes.

We have not investigated the observed phenomena that cells transfected with plasmids containing GP46A or B coding sequences produce 46 and 60kDa proteins (Figure 1, lanes 4,5). The magnitude of this size difference makes it unlikely that differential glycosylation causes the effect. Other possible explanations, including that GP46 is covalently linked via non-disulfide linkages to other macromolecules, and that production of plasmid-encoded GP46 somehow overcomes the reduced expression of chromosomal GP46 genes, need to be studied.

It is also unclear why evolution has conserved the presence of multiple GP46 genes/protein (McMahon-Pratt et al., 1992, Beetham et al., 1997). One possibility is that resistance to CML of specific vertebrates varies by GP46 gene/protein class. Our (unpublished) studies with GP46 of *L. chagasi* suggest there may be three GP46 gene families in this species, based on DNA sequences (and predicted protein coding sequences) of multiple GP46 cDNA clones. Future studies can investigate whether individual re-expression of these three GP46 classes in high passage promastigotes confers variable resistance to CML when assayed with serum derived from a range of mammals.

A mechanism for how GP46 functions in resistance of *L. chagasi* promastigotes remains to be determined in future studies. One possibility is that the heavily glycosylated GP46 prevents access of the membrane attack complex to the parasite membrane. Another possibility is that GP46 somehow causes shedding of the membrane attack complex from the parasite surface, as has peen posited for the CML-resistance activity of LPG (Puentes et al., 1990). It is also plausible that GP46, possibly through the leucine-rich repeats of the amino-region, inhibits completion of the complement cascade via protein-protein interactions (i.e. with components of the immune system). Analogous examples in which CML is inhibited by binding of parasite molecules to host complement components have been

hypothesized/implicated in *Borrelia burgdorferi*, *Escherichia coli*, *Salmonella* spp. and *Streptococcus pyogenes* (reviewed in Würzner, 1999). Further study of GP46 genes and protein, and their function in Leishmania promastigote resistance to CML can address these questions.

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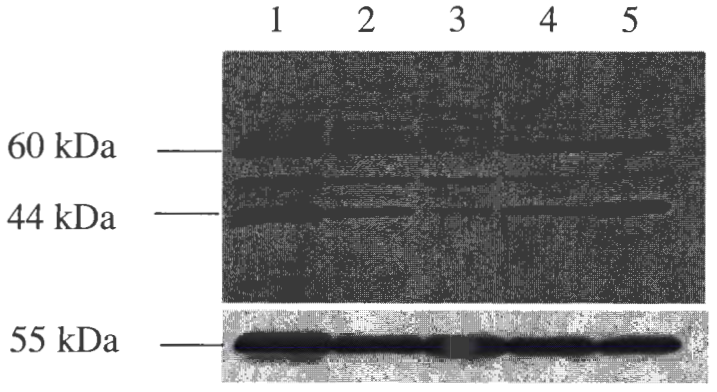

Figure 1: Expression of GP46 in transfected and non-transfected parasites
Total lysates from non-transfected promastigotes passaged for 2 weeks (lane 1), 10 weeks (lane 2), and from transfected cells passed sixteen weeks (lanes 3-5), were separated by SDS-PAGE then blotted to nitrocellulose membrane. Plasmids used for transfections contained the coding sequence for β-galactosidase, GP46A , or GP46B (lane 3, 4, or 5, respectively). Blots were probed with polyclonal antisera raised against a GST-GP46 fusion protein (upper blot), then stripped and probed with a monoclonal antisera that detects ß-tubulin (lower blot). Relative mass is shown in kDa. Following application of the appropriate secondary antisera, immuno-reactive proteins were visualized using chemiluminescence reagents (see methods).

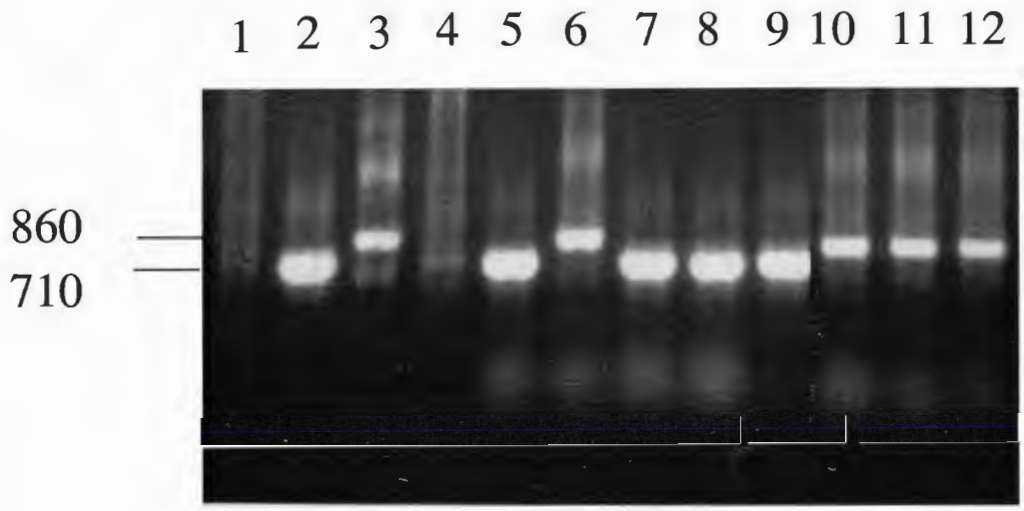

Figure 2: PCR Confirmation of Transfectants. DNA extracted from nine clonally isolated promastigotes that were transfected with plasmids containing the coding sequence for β-galactosidase (lane 4), GP46A (lanes 5, 7-9), or GP46B (lanes 6, 10-12) was amplified by PCR (see methods). PCR products were separated by agarose gel electrophoresis and visualized after ethidium bromade staining. Control PCRs included purified plasmid as template DNA: pXβGAL2 (lane 1), pXGP46A (lane 2), or pXGP46B (lane 3). Sizes of bands are indicated in base pairs.

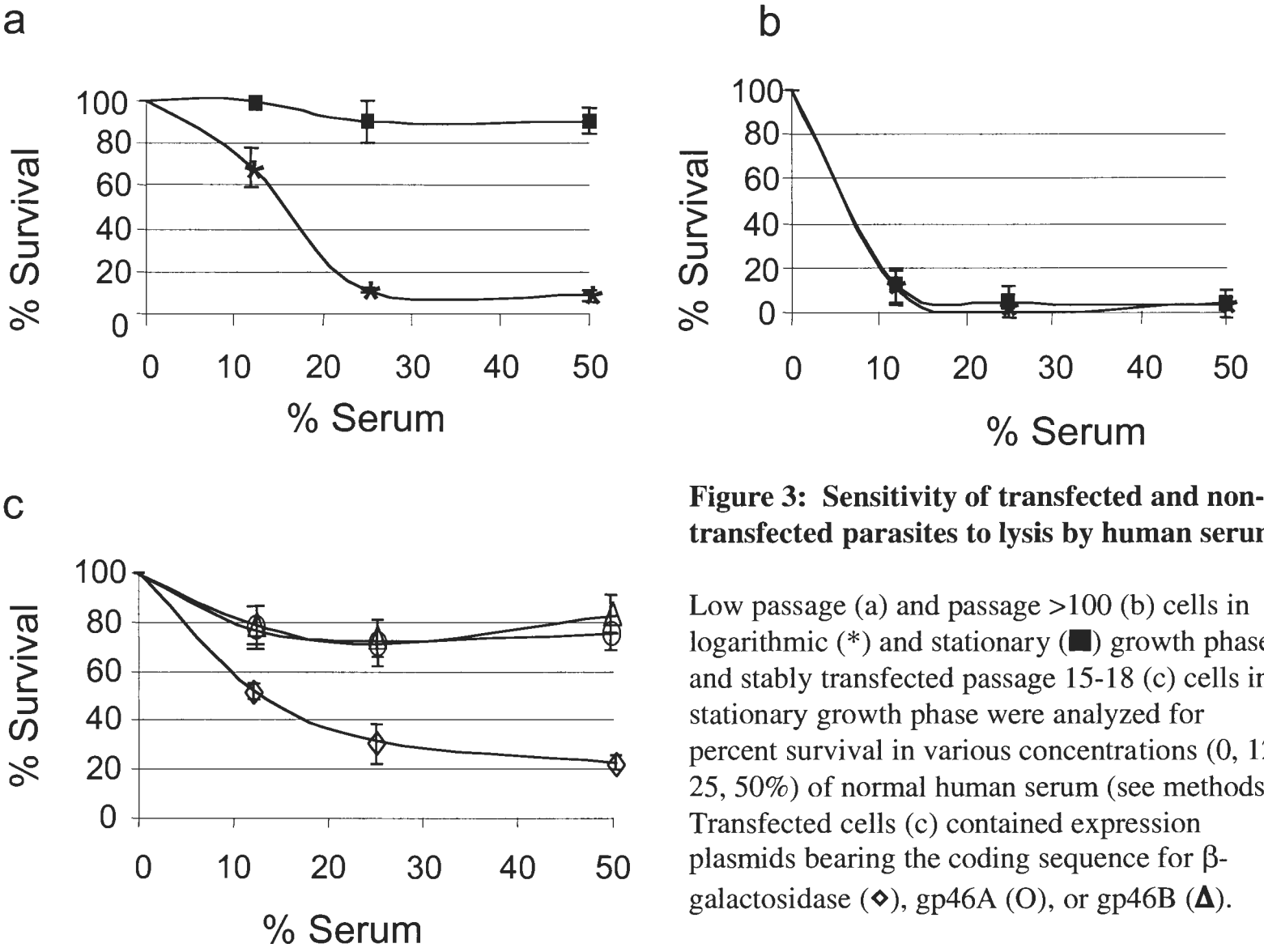

Figure 3: Sensitivity of transfected and non-transfected parasites to lysis by human serum

Low passage (a) and passage >100 (b) cells in logarithmic (*) and stationary (■) growth phases, and stably transfected passage 15-18 (c) cells in stationary growth phase were analyzed for percent survival in various concentrations (0, 12, 25, 50%) of normal human serum (see methods). Transfected cells (c) contained expression plasmids bearing the coding sequence for β-galactosidase (◇), gp46A (O), or gp46B (Δ).

CHAPTER 3. DISCUSSION

This study supports the possibility of a role for GP46 in resistance of *L. chagasi* promastigotes to complement-mediated lysis. It will be interesting to determine the mechanism of this function. A logical place to start would be to determine if GP46 binds to any complement proteins, possibly by using purified GP46 bound to a column. Protein-protein interaction with GP46 would be possible by either the leucine-rich repeat region in the amino half of the protein or the serine/threonine and acid rich carboxyl half.

Construction of the GP46-expression plasmids used here, which included the GP46 coding sequences but not any GP46-derived UTR sequences, was intended to yield plasmids that would constitutively express GP46 when transformed into recipient cells. Omission of GP46-UTRs was based on the general knowledge that in *Leishmania* spp. levels of developmentally regulated mRNAs are frequently found to be modulated by post-transcriptional mechanisms that require *cis*-acting elements contained within 3'UTR sequences (Aly et al., 1994; Nozaki and Cross, 1995; Teixeira et al., 1995; Coughlin et al, 2000, Kelly et al., 2001; Charest et al., 1996; Quijada et al., 2000, Myung et al., 2002). More specifically, studies of regulated GP46 expression have similarly determined that 3'UTR sequences confer GP46-like patterns of expression when appended to coding sequences of reporter genes (Beetham et al., 1997; Myung et al., 2002). Therefore, the observation (described below) that GP46 was not constitutively produced in high passage cells transformed with the GP46-expression plasmids was unexpected.

Figure 1 is a Western blot showing expression levels of GP46 in high passage transfectants and low passage non-transfected promastigotes in logarithmic and stationary phases of growth. No, or very little, GP46 was detected in logarithmic phase cells, including those that contained plasmids with the coding sequences of GP46 (Fig. 1a, lanes 4,5). GP46 (44 and 60 kDa bands) was detected in stationary low passage cells, and in the high passage

cells containing plasmids bearing GP46 coding sequences (Fig. 1b). Thus in the higher passage cells, expression of the GP46 coding sequence, without inclusion of GP46 5'- or 3'-UTRs in the expression plasmids, yielded the same pattern of expression (low in log phase and high in stationary phase) seen in non-transformed low passage cells.

To determine if the inability to express GP46 in logarithmic phase transfectants is due to the 3'UTR of the vector or the to open reading frame of GP46, analysis of β-galactosidase expression in the pXβGAL2 transfectants needs to be conducted. If β-galactosidase expression varies between logarithmic and stationary phases, the regulation in all transfectants is most likely due to the 3'UTR of the vector. If β-galactosidase expression in the controls does not differ between logarithmic and stationary phases, the differential regulation of GP46 in the vector is most likely due to the coding sequence of GP46, and further investigation will be called for.

Western blots that analyzed low passage stationary phase promastigotes show reaction of an antibody to GP46 with two bands migrating at 44 and 60 kDa (Fig. 1). We previously hypothesized that these two bands were the result of expression of two different GP46 genes with different coding sequences. Therefore, when high passage promastigotes were transfected with an expression vector containing a coding sequence for one GP46 gene we expected only one band to be re-expressed. Instead, transfectants produced both bands of 44 and 60 kDa regardless of which GP46 coding sequence was contained in the expression plasmid. It will be interesting to determine the nature of this observation. Possible reasons are expression of more than one form of GP46 although only one form is expressed by the plasmid, post-translational modification, or covalent bonding. Northern analysis of these transfectants will reveal if the two bands are the result of one or more GP46 mRNAs. Experiments in which the cell proteins are pulse-labeled then precipitated with GP46 antisera can determine whether one protein size chases to the other, an indication that one band derives from the other.

In *Leishmania* spp. studied, multiple non-identical genes encode GP46 (Kahl and McMahon-Pratt, 1987). Why there are multiple copies is an intriguing question. One hypothesis, based on the role of GP46 in resistance to complement-mediated lysis, is that these different versions function in resistance to CML of different mammalian species. This hypothesis can now be tested using the transfectants made here and serum from different mammals. An alternate hypothesis is that GP46 has another role within the fly gut that necessitates the diversity of genes.

This study has provided evidence of a function for GP46. These transfectants can now be used to further study the mechanism of this resistance and a reason for the existence of multiple non-identical GP46 gene copies. This will further our understanding of immune evasion and infectivity of *Leishmania* spp. that will hopefully aid the discovery of new treatments or a vaccine for this disease.

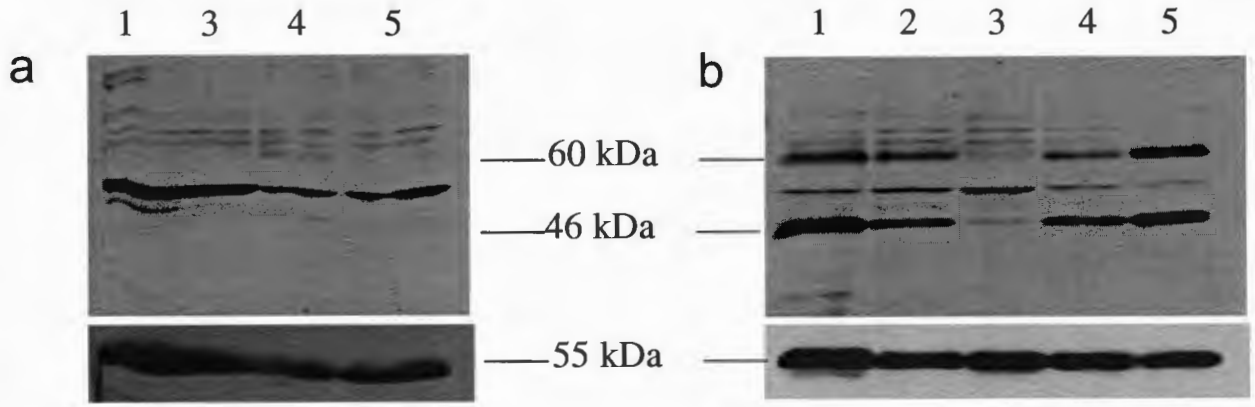

FIGURE 1: Expression of GP46 in transfected and non-transfected parasites Total lysate from non-transfected cells passaged three (lane 1) or ten weeks (lane 2), and from transfected cells passaged sixteen weeks (lanes 3-5), were separated by SDS-PAGE then blotted to nitrocellulose membrane. Cells were in logarithmic (a) or stationary (b) growth phase. Plasmids used for transfections contained the coding sequence for β-galactosidase, GP46A , or GP46B (lane 3, 4, or 5, respectively). Blots were probed with polyclonal antisera raised against a GST-GP46 fusion protein (upper panel), then stripped and probed with a monoclonal antisera that detects ß-tubulin (lower panel). Relative mass is shown in kDa. Following application of the appropriate secondary antisera, immuno-reactive proteins were visualized using chemiluminescence reagents (see methods).

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