Processing and trafficking of GP63 in *Leishmania mexicana GPI8* mutants

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Abstract

GPI8 is a Clan CD, family C13 cysteine protease and the catalytic core of the GPI: protein transamidase (GPIT) complex. GPI8 catalyses the addition of pre-formed glycosylphosphatidylinositol (GPI) anchors to the C-terminus region of GPI-anchored proteins. The GPIT complex has been well characterised in higher eukaryotes, and contains at least 4 components. In the parasitic protozoon *Leishmania mexicana* GPI8 is non-essential. Δ*gpi8* mutants lack GPI-anchored GP63 from the cell surface.

Site-directed mutagenesis of *L. mexicana GPI8*, followed by expression in the Δ*gpi8* cell line, identified the active site cysteine (C216) and histidine (H174) residues. The amino acid C94 was also identified as a functionally important residue. Mutation of the amino acid H65 had no detectable effect on GPI8 activity. Re-expression of $GPI8^{C216G}$ within WT cells had a dominant negative effect on the processing and trafficking of GP63 to the cell surface. This indicates that GPI8 is part of a larger complex within *L. mexicana*. Attempts to identify other components of the GPIT complex, by epitope tagging, or the production of a GPI-anchored GFP were unsuccessful.

The Δ*gpi8* cell line was used to study the effect that a GPI-anchor deficiency has on the processing and trafficking of a GPI-protein. Non-GPI-anchored GP63 was secreted from the Δ*gpi8* cell line, and this secreted form was not processed in the same way as in WT cells. In Δ*gpi8* non-GPI-anchored GP63 was glycosylated and secreted without further processing from the cell with a $t_{1/2}$ of 120 minutes. Loss in GPI anchoring did not result in the intracellular retention of GP63 as found in mammalian cells. N-glycans were shown to be important for the secretion of GP63 in the absence of a GPI anchor. In WT cells the majority of GP63 was rapidly glycosylated, GPI-anchored and trafficked to the surface with defined processing intermediates. WT cells secreted 2 isoforms of GP63 into the medium with different kinetics. The 65s isoform was not GPI-anchored, while the 63s isoform retained its anchor. It is suggested that anchored and unanchored GP63 are trafficked via 2 different pathways in *Leishmania mexicana*; a classical pathway whereby GP63 is N-glycosylated, GPI-anchored and then undergoes further modification during transport to the cell surface; or a direct secretion pathway whereby non-anchored GP63 is secreted from the cell without modification.

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aa	Amino acid
BFA	Brefeldin A
ConA	Concanavalin A
CRD	Cross-reacting determinant
DABCO	1,4-diazobicyclo[2.2.2.]octate
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
Dol-P-Man	Dolichol phospho mannose
DPMS	Dolichol phospho mannose synthase
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
ER	Endoplasmic reticulum
EtN-P	Ethanolamine phosphate
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GIPL	Glycoinositolphospholipid
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosaminyl
GlcN-PI	Glucosaminyl-Phosphatidylinositol
GPI	Glycosylphosphatidylinositol
GPIT	GPI: protein transamidase
LPG	Lipophosphoglycan
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PIG	Phosphatidylinositol glycan
PI-PLC	Phosphatidylinositol phospholipase C
PNGaseF	Peptide: N-Glycosidase F

RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TGN	Trans-Golgi network
Tris	Tris(hydroxymethyl)amino methane
TX-114	Triton X 114
UV	Ultraviolet
VSG	Variant surface glycoprotein
WT	Wild type

Measurements

bp	base pairs
kb	kilobase pairs
Ci	Curie
Da	daltons
kDa	kilodaltons
µg	micrograms
mg	milligrams
ng	nanograms
M	molar
mM	millimolar
µM	micromolar
pM	picomolar
µl	microlitres
ml	millilitres
g	G-force (centrifugation)
V	volts

Declaration

I declare that the work presented in this thesis is my own except where otherwise stated.

Miriam Ellis

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Chapter 1

General Introduction

1.1 Trypanosomatids

1.1.1 Introduction

The trypanosomatids are parasitic protozoa, many of which cause a range of parasitic diseases in mammals. These diseases have a severe social and economic impact on the areas of the world in which they are prevalent. Species of trypanosomatids include *Trypanosoma brucei* spread by the tsetse fly and *Leishmania* spread by the sandfly. Sub-species of *T. brucei* cause the serious wasting diseases known as nagana in cattle, or African sleeping sickness in humans, which often leads to death if untreated. African trypanosomes are endemic in 36 countries across Central and West Africa. *Leishmania* species cause a variety of diseases, which can lead to severe disfigurement or death. *Leishmania* has a worldwide distribution encompassing Southern Europe, Asia and Africa (old world) and North and South America (new world). *Leishmania* is a diamorphic parasite, with a lifecycle which alternates between the sandfly vector and mammalian hosts, which includes humans (Handman, 1999; Handman, 2001).

1.1.2 Leishmaniasis

There are approximately 20 human infective species and sub-species of *Leishmania* which are the causative agent of a variety of human diseases collectively known as the leishmaniases. The varying clinical manifestations of the disease can be divided into four categories (Handman, 2001).

Visceral leishmaniasis also known as kala azar, is the most serious condition, and can cause 100% mortality if left untreated. It is characterised by irregular bouts of fever, swelling of the spleen and liver, weight loss and anaemia. 90% of cases of visceral leishmaniasis occur in Bangladesh, Brazil, India and Sudan.

Species; *L. donovani, L. tropica.*

Cutaneous leishmaniasis is the most common form of the disease, and causes skin lesions and ulcers which self heal. The disease can cause a large number of lesions

(over 200), usually on exposed areas such as the face, arms and legs, and can cause permanent disability and severe disfigurement.

Species; *L. major*, *L. mexicana*, *L. tropica*, *L. aethiopica.*

Mucotaneous leishmaniasis initially produces skin ulcers, which spread to the mucose membranes of the nose mouth and throat. Here it can cause massive tissue destruction, leading to dreadful facial disfigurement and disability.

Species; *L. braziliensis.*

Diffuse cutaneous leishmaniasis produces chronic skin lesions, which do not heal, and are difficult to treat.

Species; *L. mexicana*, *L. aethiopica.*

In general the extent of disease progression depends on the species initiating infection, however the general health and immunological competence of the infected individual also effects the success of infection.

Leishmaniases infections occur in 88 countries world wide, most of which are developing countries, and 350 million people are at risk from infection. Figures from the World Health Organisation estimate that 12 million people are infected, and 2 million new cases are estimated to occur annually (WHO, Leishmaniasis control home page: http://www.who.int/ctd/html/leis.html). Epidemics of both visceral and cutaneous leishmaniasis occur frequently. An epidemic of visceral leishmaniasis in Sudan in the 1990s was estimated to have killed 100,000 people (McGregor, 1998). At present an epidemic of cutaneous leishmaniasis in Afghanistan is estimated to have infected 200,000 people (WHO website). In recent years there has been an increase in both the number of cases and the geographic spread of the disease. This is thought to be in part due to co-infection with HIV. In southern Europe 1.5% to 9% of AIDS suffers also suffer from newly acquired or reactivated visceral leishmaniasis (Alvar *et al.*, 1997).

The prevention of infection is an important aspect in the control of the disease. Strategies include the control of the sandfly vector by the spraying of insecticides, and the control of the reservoir host. Large scale programmes are often difficult to implement in developing countries, or inappropriate in the countryside areas where the disease is prevalent. No vaccine is at present available (Handman, 2001). In many developing countries a common method of protection from cutaneous leishmaniasis, is

the deliberate infection of babies, providing immunisation and preventing the occurrence of disfiguring scars on the face.

Treatment of the disease relies on the use of chemotherapeutic agents. Many of the drugs currently in use are in themselves toxic, producing an array of unwanted side effects. They also require intravenous delivery, and are prohibitively expensive for use in developing countries. In recent years there has also be an increase in parasite drug resistance (Bryceson, 2001). Traditionally treatment has required a long, high dosage course of treatment with toxic, pentavalent antimony based drugs, such as sodium stibogluconate, or meglumine antimonate. Increased drug resistance to antimonials, has resulted in the use of amphotericin B, which inhibits the biosynthesis of ergosterols, and therefore leads to the membrane permeability of the parasite. Amphotericin B is toxic and in some cases can lead to death. Pentamidine is also used, but has increasingly been found to be ineffective, due to parasite drug resistance, and causes a number of serious side effects including diabetes, and death (Sundar, 2001). More recently miltefosine has been developed as an anti-leishmanial, and affects cell signalling pathways and membrane synthesis. The drug has the advantage of being taken orally (Fischer *et al.*, 2001). The rapid increase in parasite drug resistance means that the development of further new effective drugs is essential to allow the use of effective multi-drug regimes. The urgent need for the development of an effective vaccine or isolation of new drug targets, are important factors in the study of this parasite.

1.1.3 The lifecycle of *Leishmania*

The lifecycle of *Leishmania* is diamorphic, cycling between the sandfly vector and the vertebrate host (Figure 1.1). *Leishmania* have three distinct morphological forms. In the gut of the insect host, *Phlebotomus* female sandflies, they exist as flagellated procyclic promastigotes. The protozoa are attached to the gut wall and rapidly divide by binary fission. From the gut they migrate to the sandfly mouthparts. Here they undergo a morphological change to become metacyclic promastigotes, that is, motile, non-dividing and infective to the mammalian host.

Infection of the mammalian host occurs from an infected sandfly bite, metacyclic promastigotes are injected directly into the bloodstream. Activation of the alternative

complement pathway results in the phagocytosis of the protozoa by macrophages (Brittingham and Mosser, 1996). Inside the macrophage, the phagolysosome fuses with lysosomes to form parasitophorous vacuoles. The protozoa differentiate into their third morphological form, non-motile dividing amastigotes. Amastigote numbers rapidly increase until the macrophage undergoes an oxidative burst, releasing the amastigotes directly into the bloodstream to infect other macrophages. This process continues, until a sandfly takes up infected macrophages during a bloodmeal. The amastigotes emerge from the macrophage and differentiate to the procyclic promastigote form within the insect gut.

1.2 Surface Molecules

The *Leishmania* lifecycle is complex, requiring the parasite to adapt rapidly to the different conditions within the insect gut and mouthparts, and the macrophage within the mammalian host. The parasite must also evade the mammalian immune response, while exploiting its opsonic properties. The architecture of the parasite's surface coat is particularly important in maintaining the organism's survival. GPI-anchored proteins and glycoconjugates cover the cell surface of trypanosomatids, and these are thought to play a key role in parasite survival. The major cell surface GPI glycoconjugates of *Leishmania* fall into three distinct classes; glycosylphosphatidylinositol (GPI) anchored proteins, lipophosphoglycans (LPG), and glycoinositolphospholipids (GIPLs) (Figure 1.2).

1.2.1 GPI-anchored proteins

Outer surface proteins are attached to the membrane either by transmembrane domains, or modification with a glycosylphosphatidylinositol (GPI) anchor, whereby proteins are anchored to the membrane via covalent linkage to phosphatidylinositol (PI). The structure of the GPI anchor was first described for the variant surface glycoprotein (VSG), of *T. brucei* (Ferguson *et al.*, 1985; Ferguson *et al.*, 1988). Since then over 200 examples of GPI-anchored proteins have been identified in eukaryotes, and analyses of the known GPI anchor structures demonstrate that they each have a conserved core structure, which is further modified in a protein and species specific manner (Ferguson, 1999). The conserved GPI core structure is comprised of the protein linked at the C-terminal end via ethanolamine phosphate (EtN-P) to the glycan backbone: Manα1-

2Manα1-6Manα1-4GlcNH$_2$. The glycan backbone is linked to the inositol ring of phosphatidylinositol (PI) (Figure 1.2) (McConville and Ferguson, 1993). Variations from this core include: i) The substitution of the tetrasaccharide backbone with elaborate side chains, an example being the Variable Surface Glycoprotein (VSG) of *T. brucei*, which has highly variable and complex additions. ii) Addition of the fatty acid palmitate to the inositol ring, this is particularly true of mammalian GPI anchors. iii) Lipid moieties are highly variable, and yeast anchors commonly contain ceramide. iv) Higher eukaryotes have additional EtN-P residues on the mannose backbone (Reviewed (McConville and Ferguson, 1993).

In *Leishmania* the major GPI-anchored proteins include GP63 (also known as major surface protease (MSP) or leishmanolysin) (Bouvier *et al.*, 1985), and GP46, also referred to as promastigote surface antigen 2 (PSA2) (Murray *et al.*, 1989). The structure of the GPI anchor of GP63 is the same as the generalised core structure described above, and is not modified further (Schneider *et al.*, 1990). GP63 is described in more detail in section 1.3.

GP46 is abundant on the cell surface of all species of *Leishmania* promastigotes, with the exception of *L. braziliensis* (McMahon-Pratt *et al.*, 1992). GP46 is encoded by a polymorphic multigene family (Symons *et al.*, 1994). Its function is unknown but has been shown to contain leucine rich 24 amino acid repeats (Lohman *et al.*, 1990). GP46 is down-regulated in amastigotes, as assessed by mRNA levels (Handman *et al.*, 1995). Similarly the protein level of GP63 is also greatly decreased in amastigotes (Bahr *et al.*, 1993). In *L. chagasi* the mRNA levels of both GP46 and GP63 show a 30 fold increase as the promastigotes enter the infective stationary phase (Myung *et al.*, 2002).

1.2.2 Proteophosphoglycans (PPG).

Proteophosphoglycans (PPGs) are a group of proteins which are modified by phosphoglycans. The proteins consist of a polypeptide backbone consisting of serine/ alanine/ proline repeat domains, the serine residues are extensively modified by complex, and highly variable phosphoglycan side chains, which can extend to up to 30 sugar residues in length (Ilg, 2000b). The PPG molecules include secreted acid phosphatase, a non-filamentous proteophosphoglycan, and a filamentous proteophosphoglycan. A GPI-anchored form of PPG has also been identified, which is

present on the cell surface of both *L. major* promastigotes and amastigotes (Ilg *et al.*, 1999). The precise function of PPGs is not known, but they are thought to be involved in macrophage interaction and invasion, and the formation of parasitophorous vacuoles (Piani *et al.*, 1999; Foth *et al.*, 2002). Study of a *L. mexicana* mutant cell line, Δ*lpg2*, showed that these cells lacked both PPG and LPG from the cell surface, but were still able to invade macrophage and infect mice (Ilg *et al.*, 2001).

1.2.3 Lipophosphoglycans (LPG)

The surface coat of promastigotes is dominated by LPGs, which are complex carbohydrates attached to the cell membrane via a GPI anchor. LPGs are highly variable, but the basic structure includes an extended phosphoglycan chain, made up of phosphosaccharide repeats and a terminating cap structure, and a GPI anchor. This anchor is structurally distinct from the protein-linked GPI anchor, and contains a lysoalkyl-PI. The LPG anchor contains the common GPI-protein anchor motif Manα1-4GlcN-PI, but a second mannose residue is linked via a α1-3 bond instead of the α1-4 bond present in the GPI protein anchor. The second mannose residue is linked to a galactosefuronose residue, which in turn is linked to two galactose residues. The final galactose residue is linked to the phosphosaccharide repeat unit. The mannose residues are often modified with an additional side chain (McConville and Ferguson, 1993).

LPGs are present on the surface of *Leishmania* promastigotes at a level of $5x10^6$ molecules per cell (McConville and Blackwell, 1991). LPGs are also present on the surface of amastigotes, though at a vastly reduced level, and in a modified form (Moody *et al.*, 1993). During the lifecycle the length of the phosphoglycan chains alters, for example, the differentiation of *L. major* procyclic promastigotes to metacyclic promastigotes corresponds with the doubling in length of the phosphoglycan chain, resulting in an increase in thickness of the glycocalyx (McConville *et al.*, 1992).

LPGs act as a ligand for the attachment of procyclic promastigotes to the midgut wall of the sandfly vector, and may also protect promastigotes from the digestive enzymes within the gut (Sacks *et al.*, 1994; Sacks *et al.*, 2000). LPGs are also thought to confer resistance to complement mediated lysis, and facilitate uptake of the parasite into macrophages via interaction with complement receptors (Puentes *et al.*, 1990).

Leishmania strains with mutations in the LPG biosynthetic pathway have been used to assess the requirements for LPG in *Leishmania* infections. An *L. major* LPG minus strain showed decreased virulence within the sandfly vector, mammalian macrophage, and mouse (Sacks *et al.*, 2000; Späth *et al.*, 2000). However similar studies with an *L. mexicana* LPG minus strain, demonstrated that the mutants replicated normally in macrophage and were able to infect mice (Ilg, 2000a). It is suggested that this disparity in the requirement for LPG reflects the divergence between different *Leishmania* species (Turco *et al.*, 2001).

1.2.4 Glycoinositolphospholipids (GIPLs)

The GIPLs are free GPI anchor structures which are abundant in both promastigote and amastigote lifecycle stages. The GIPLs have a variety of structures: Type 1 GIPLs have the same glycan core structure as the GPI protein anchor. Type 2 GIPLs have the same core structure as the LPG anchor. Hybrid-Type share similarities in common with each (Figure 1.2, panel B) (McConville and Ferguson, 1993). The GIPLs may also be modified with distinct lipid moieties, ethanolamine phosphates, and glycans (McConville *et al.*, 1993; Ralton and McConville, 1998).

The GIPLs are abundant in both promastigote, and amastigote lifecycle stages and are present on the cell surface at an estimated 10^7 molecules per cell (McConville and Blackwell, 1991). As the most dominant molecule on the amastigote cell surface they are thought to act as a protective glycocalyx within the parasitophorous vacuole (Winter *et al.*, 1994). A *L. amazonensis* strain expressing Phospholipase C from an episome, was demonstrated to have a decreased number of GIPLs present in the amastigote form. This strain showed decreased virulence in hamster infections, and in *in vitro* macrophage infections differentiation of promastigotes into amastigotes resulted in growth arrest (Mensa-Wilmot *et al.*, 1999). Dolicholphosphate-Man synthase (DPMS) is an enzyme required for the formation of Dolicholphosphate-Man (Dol-p-Man) which is directly utilised in the biosynthesis of glycoconjugates. A *L. mexicana* Δ*dpms* cell line deficient in this enzyme, was unable to express GIPLs, LPGs or GPI anchored proteins on the cell surface (Garami *et al.*, 2001). However this cell line was still able to infect macrophage and mice, though with reduced virulence.

Phosphomannose mutase (PMM) is an enzyme required for the activation of mannose, and is one of the first enzymes in the mannose activation pathway. An *L. mexicana* Δ*pmm* cell line was unable to express any detectable mannose containing GIPLs, LPGs, GPI-anchored proteins, or PPG on the cell surface. This Δ*pmm* cell line was avirulent, as it failed to infect macrophages or mice (Garami *et al.*, 2001). It has therefore been suggested that whilst all classes of glycoconjugates are important virulence factors in *Leishmania*, a certain amount of redundancy exists. Cell lines defective in only one or two classes of surface expressed glycoconjugates are still infective to mice. It is only the loss of all classes of glycoconjugates from the cell surface which results in an avirulent phenotype (Garami *et al.*, 2001).

The characterisation of biosynthesis intermediates from GPI anchors, LPGs and GIPLs suggests that the LPG and protein anchors appear to share a set of common early intermediates. They may share a common early biosynthesis pathway which diverges at the point of the addition of the second mannose residue (Ralton and McConville, 1998). GIPLs however share no common intermediates and utilise an alternate PI species. GIPL biosynthesis also seems to occur at a higher rate than either LPG, or protein anchor biosynthesis. The presence of three individual pathways for glycoconjugate biosynthesis would allow the independent regulation of expression of the different molecules at different lifecycle stages (Ralton and McConville, 1998). The biosynthetic pathway of GPI protein anchors is described in section 1.4.

It can be seen that during the different lifecycle stages the *Leishmania* surface coat undergoes a series of changes. The promastigote form is dominated by GIPLS, LPGs, and GPI-anchored proteins, whilst the amastigote form sees a down regulation of both LPGs and GPI-anchored proteins, but retains the GIPL layer. These surface glycoconjugates are considered to be extremely important for parasite virulence.

1.3 GP63

1.3.1 Genetics and stage regulated expression

The major GPI-anchored protein of *Leishmania* promastigotes is the 63kDa protein, GP63 (Bouvier *et al.*, 1985; Russell and Wilhelm, 1986). It is estimated to be 1% of the total protein in the cell, and is present at $5x10^5$ molecules per parasite (Medina-Acosta

et al., 1989; Bahr *et al.*, 1993). GP63 is also present in the amastigote stage of the lifecycle (Medina-Acosta *et al.*, 1989; Frommel *et al.*, 1990), though at a reduced level. In *L. major* amastigotes GP63 proteolytic activity has been estimated to represent less than 1% of that detected in promastigote cells (Schneider *et al.*, 1992). Labelling of total *L. mexicana* cell lysates suggests that GP63 expression in amastigotes is 10% of that found in promastigotes (Medina-Acosta *et al.*, 1989; Bahr *et al.*, 1993). Both mRNA and protein levels increase four hours after the onset of differentiation of amastigotes into promastigotes, demonstrating stage regulated expression (Schneider *et al.*, 1992).

In all species of *Leishmania* studied, GP63 is encoded by a series of multi-copy, tandemly repeated genes (Button *et al.*, 1989; Webb *et al.*, 1991; Medina-Acosta *et al.*, 1993). The genomic organisation of the seven genes encoding GP63 in *L. major* has been well characterised (Button *et al.*, 1989; Joshi *et al.*, 1998; Voth *et al.*, 1998). Five homologous 1.8 kb genes (genes 1-5) are present in a tandem array, each separated by a 1.3 kb intergenic region. Gene 6 is 8 kb downstream from gene 5, and is less highly conserved. Gene 7 is 1.7 kb downstream from gene 6 (Voth *et al.*, 1998: Joshi *et al.*, 1998). The genes are regulated stage specifically. Genes 1-5 are expressed exclusively in promastigote stage parasites, gene 6 is expressed throughout the life cycle, and gene 7 is expressed only in stationary-phase promastigotes, and amastigotes (Joshi *et al.*, 1998; Voth *et al.*, 1998). Genes 1-5 are highly conserved (Button *et al.*, 1989), and encode for proteins which are GPI-anchored (Schneider *et al.*, 1990). The predicted protein for *L. major* Gene 6 is less well conserved in its C-terminal GPI signal attachment site than the GPI-anchored proteins encoded by genes 1-5 (Voth *et al.*, 1998). It was speculated that this protein may have a transmembrane domain and not be GPI-anchored. Expression of gene 6 in *L. donovani*, radio-labelling with myristic acid, and immune-precipitation with a *L. major* specific GP63 antibody demonstrated that the protein was GPI-anchored (Voth *et al.*, 1998). Sequence analysis suggests that gene 7 is identical to gene 1, which encodes a GPI-anchored protein (Voth *et al.*, 1998).

The genomic organisation of GP63 in *L. mexicana* has been shown to be more complex (Medina-Acosta *et al.*, 1993). GP63 is encoded by an estimated 11 genes, at a single chromosomal locus. These are grouped into three sub-classes based on restriction site

polymorphism (C1, C2, and C3). Four GP63 C2 genes are upstream from a cluster of five C1 genes. A single C3 gene is 3' to the C1 cluster. Northern blotting with class-specific probes demonstrated that the C1 subclass is expressed in amastigotes, and at a lower level in promastigotes. C2 and C3 are expressed at high levels in promastigotes only (Medina-Acosta *et al.*, 1993). The predicted protein sequence for a clone from the C1 subclass showed considerable variation from *L. major* GP63 encoded by gene 1. The *L. mexicana* sequence has nine potential N-glycosylation sites, compared to three sites predicted in *L. major* GP63. The C-terminus of the *L. mexicana* C1 GP63 is extended and does not appear to contain sequence compatible with GPI anchor addition. Therefore this *L. mexicana* amastigote form of GP63 is predicted to be non GPI-anchored (Medina-Acosta *et al.*, 1993). Microscopy and labelling studies on *L. mexicana* suggest that only a small sub-population of the detectable amastigote GP63 is GPI-anchored, the remainder is found intracellularly (Medina-Acosta *et al.*, 1989; Bahr *et al.*, 1993). Cleavage of GPI anchors from proteins with phosphatidylinositol-specific phospholipase C (PIPLC) produces a carbohydrate epitope on the C-terminus of the protein, which is recognisable by the cross-reacting determinant (CRD) antibody. The antibody can therefore be used for determining if soluble proteins have previously been GPI-anchored. Soluble *L. mexicana* promastigote GP63 is recognised by the CRD antibody, while soluble amastigote GP63 is not. This provides further evidence that the amastigote form of GP63 in *L. mexicana* is not GPI-anchored (Ilg *et al.*, 1993). Electron-microscopy localised the intracellular soluble GP63 to lysosomes (Bahr *et al.*, 1993).

In *L. chagasi*, GP63 (termed msp for major surface protease) is encoded by more than 18 genes located in a 80kb cluster (Roberts *et al.*, 1993). These are divided into three classes and named according to the lifecycle stage in which they are expressed: logarithmic (mspL), stationary (mspS) and constitutive (mspC) (Roberts *et al.*, 1993). They are categorised by unique sequences present in the 3' untranslated regions (UTR) (Ramamoorthy *et al.*, 1992). The genes are organised such that four tandem stationary phase genes (*msp*S2, *msp*S1, *msp*S3, and *msp*S5) are followed by 12 or more logarithmic genes, one constitutively expressed gene, and a stationary phase gene (*msp*S4) (Roberts *et al.*, 1993).

Regulation of the stage-specific expression of GP63 is thought to be post-transcriptional (Ramamoorthy *et al.*, 1995). In *L. major* the coding and intergenic regions of genes 1-7 are conserved, but the 3'UTR are divergent (Voth *et al.*, 1998). Expression of the marker gene Thymidine Kinase (TK) from a plasmid, could be regulated to specific stages of the *L. mexicana* lifecycle by cloning in different *L. major* GP63 intergenic regions 3' to the gene. The 3' UTR from *L. major* GP63 gene 3 resulted in TK RNA and protein expression in promastigote cells only. The 3'UTR from *L. major* GP63 gene 6 resulted in TK RNA and protein expression in both lifecycle stages. This suggests that stage-specific expression of GP63 is regulated by the 3'UTR (Kelly *et al.*, 2001).

Mechanisms for controlling the stage-regulated expression of GP63 have been examined in detail in *L. chagasi*. Genes from all classes of GP63 are constitutively transcribed in *L. chagasi*, suggesting that the stage-regulated expression of the proteins is controlled post-transcriptionally (Ramamoorthy *et al.*, 1995). A construct was produced in which the entire 3'UTR of an mspS gene, including the intergenic region (IR) and the region 5' of the downstream gene, was placed downstream of the reporter gene. The reporter protein was expressed in a growth-specific manner similar to that of the mspS class of GP63 (Ramamoorthy *et al.*, 1995). This demonstrated that regulatory sequences were present in this region. Sequence analysis identified an ORF within this region, termed *mag* (*msp* associated gene), and it was demonstrated that elements were required from each of the 3'UTR, IR, and *mag* to regulate GP63 expression (McCoy *et al.*, 1998). Comparison of sequences of the 3'UTR from the genes encoding GP63 (class mspS), and a second stage regulated protein PSA (GP46) identified common segments (Myung *et al.*, 2002). The systematic deletion of these segments within the *msp*S 3'UTR, and the effects of these deletions on the expression of a reporter protein, was used to identify regulatory elements within this region. However, the complex nature of the regulation suggested that a more detailed analysis of the 3'UTR was required (Myung *et al.*, 2002).

In general, the multiple genes encoding GPI-anchored forms of GP63 are highly conserved both within and between species, and the protein has several domains characteristic of a GPI-anchored metalloproteinase (Button and McMaster, 1988). The

domain structure of GPI-anchored GP63 can be summarised: an ER-signal direction sequence is present at the amino terminal end, and is adjacent to a regulatory pro-region, a GPI anchor addition site and a hydrophobic tail are present at the COOH-terminal end. These three domains are cleaved off at different times during the trafficking and processing of the protein (Button and McMaster, 1988; McMaster *et al.*, 1994).

1.3.2 Metalloproteinase activity.

L. major GP63 has been characterised as a zinc metalloproteinase, associating with one atom of zinc per molecule of GP63 (Bouvier *et al.*, 1989). It is active in a neutral to alkaline pH range (pH 7-10), and is site specific in its proteolytic activity, cleaving on the amino side of serine and threonine. It shows significant inhibition with the chelator 1-10 phenanthroline (Ip *et al.*, 1990). The amastigote *L. mexicana* GP63 has a pH optimum which has been shifted to the acidic range (pH 5.5-6) (Ilg *et al.*, 1993), which is consistent with a lysosomal location. The protein sequence of *L. major* GP63 shows sequence identity to other zinc metalloproteinases, such as thermolysin, at the proposed active site, HEXXH. The histidine residues act as the zinc-binding domain, and the glutamic acid is the catalytic active site residue (Bouvier *et al.*, 1989; McMaster *et al.*, 1994). Site-directed mutagenesis produced an inactive protein confirming glutamic acid as the catalytic active-site residue (McMaster *et al.*, 1994; Macdonald *et al.*, 1995; McGwire and Chang, 1996). The crystal structure of promastigote *L. major* GP63 has been solved, and the N-terminal domain is similar in structure to that of the catalytic modules of other zinc proteases (Schlagenhauf *et al.*, 1998).

Metalloproteinases are synthesised in an inactive form, and activated by a 'cysteine switch' mechanism (Springman *et al.*, 1990). Latency is maintained by obstruction of the active site by the protein's regulatory pro-region. A cysteine residue within the pro-region binds to the zinc atom in the catalytic active site, disruption of this complex results in an active enzyme. A recombinant form of GP63 (reGP63) lacking its GPI signal was expressed and secreted using a baculovirus expression system (Button *et al.*, 1993). This recombinant protein was latent, and was of the predicted size of the mature protein and pro-region. It was activated by treatment with $HgCl_2$ (Button *et al.*, 1993). $HgCl_2$ activates latent metalloproteinases by disrupting the cysteine-zinc complex

(Springman *et al.*, 1990; Grant *et al.*, 1992). A non-GPI-anchored form of GP63 was expressed and secreted from Cos-7 cells, and this protein was also shown to be inactive (McMaster *et al.*, 1994; Macdonald *et al.*, 1995). Activation of this protein corresponded with a decrease in protein size, sequencing of the different-sized protein intermediates showed this size decrease was a result of the partial loss of the pro-region. The activation of the protein corresponded to the loss of Cys48 from the pro-region, and pro-region removal is therefore involved in GP63 activation, which is similar to the situation found in higher eukaryotes (Macdonald *et al.*, 1995). Incubation of latent reGP63 with previously activated enzyme did not activate the latent reGP63, suggesting the activation mechanism was not autocatalytic (Macdonald *et al.*, 1995). The Cos-7 transient expression system was also used to produce two recombinant forms of GP63, which were membrane-associated. GPI-GP63 retained the protein's GPI-anchor attachment signal whilst a second GP63 was engineered to contain a trans-membrane domain (Macdonald *et al.*, 1995). Both membrane-associated proteins were active on the surface of Cos-7 cells, compared to the secreted form of GP63 that was inactive. Activation was not thought to be directly linked with the GPI-anchor addition pathway, but was speculated to be mediated by enzymes localised in a membrane trafficking pathway (Macdonald *et al.*, 1995).

Activation of secreted and GPI-anchored forms of GP63 was also examined directly in *Leishmania* (McGwire and Chang, 1996). A strain of *L. amazonensis*, described as GP63 deficient, was obtained by continuous growth of promastigotes *in vitro* for 3 years (Kink and Chang, 1988). The strain was found to have decreased virulence, a decrease in glycosyltransferase activity, and had three fold less GP63 expression and activity when compared to virulent cells. This 'GP63-deficient' strain was transfected with a plasmid construct containing a *L. major* GP63 mutated at the anchor addition site. The mutated GP63 was shown to exit from the cell into the medium as two sized forms, 65kDa, and 63kDa. Cell lysates from cells expressing the mutated form of GP63, or the *L. major* WT GPI-anchored form, were treated with PI-PLC, and western blotted with the CRD antibody. The CRD antibody detected the *L. major* WT protein but no CRD epitope was detected in the proteins mutated at the anchor addition site. This demonstrated that the mutated GP63 protein did not receive a GPI anchor (McGwire and Chang, 1996). GP63 is a protease, and in its active form digests gelatin.

Assessment of gelatinolytic activity can be used to assess GP63 activation. The secreted forms of GP63 had gelatinolytic activity, suggesting these proteins were active (McGwire and Chang, 1996). This result contrasts with the results found using the Baculovirus and Cos-7 expression systems (Button *et al.*, 1993; McMaster *et al.*, 1994; Macdonald *et al.*, 1995). It was suggested that GPI-anchoring does not affect the activation and catalytic activity of GP63 (McGwire and Chang, 1996).

1.3.3 Glycosylation

N-linked glycans are complex structures, in which the basic core structure is synthesised by a glycan biosynthetic pathway. The core is added to the protein by the oligo-saccharide transferase (OST) during translocation of the protein into the ER (Johnson and van Waes, 1999). Further modifications and additions to the glycan occur as the glycosylated protein moves through the ER and Golgi apparatus, a process which has been well studied in higher eukaryotes (Dwek, 1996). Tunicamycin is an inhibitor of UDP-GlcNAc:dolichol phosphate GlcNac-1-P transferase (GPT), the first enzyme in the glycan biosynthetic pathway, and therefore is an inhibitor of N-linked glycan formation.

L. major GP63 has three potential N-linked glycosylation sites as identified from sequence analysis (Button *et al.*, 1989), each of which has been demonstrated by mutagenesis studies to be N-glycosylated (McGwire and Chang, 1996). Four major different oligosaccharide structures were identified from *L. mexicana amazonensis* GP63, and these were all of the biantennary oligomannose form (Olafson *et al.*, 1990).

The glycosylation of GP63 was further examined by using recombinant forms of *L. major* GP63 expressed in Chinese hamster ovary (CHO) cells (Morrison *et al.*, 2000). GP63 was either expressed as a GPI-anchored form, or modified for secretion, by truncation of the C-terminus. The glycan structures of both forms of protein were similar, complex biantennary types. However, differences were observed in the profiles of the glycans on the GPI-anchored GP63, compared with the secreted form, and these were shown to be due to differences in glycan modification. Pulse-chase labelling suggested that the dynamics of the secretion and membrane pathways were similar, both proteins were detected extracellularly after 60 minutes, and both proteins became resistant to EndoH treatment after 40 and 60 minutes. EndoH sensitivity is a marker for

proteins passing through the Golgi. Processing of glycans in the medial compartment of the Golgi confers EndoH resistance, whilst unprocessed glycans remain sensitive. Alteration of recombinant GP63 for secretion in mammalian cells affects its glycan modifications. Differences in modifications to the N-linked glycans were considered to be most likely due to changes in protein conformation, and therefore the proteins accessibility to glycosyl-transferases (Morrison *et al.*, 2000).

Growth of wild type *L. major*, and *L. mexicana* promastigotes in medium containing tunicamycin resulted in the production of a smaller-sized GP63 protein (Funk *et al.*, 1994). This non-glycosylated protein was demonstrated by biotinylation and PI-PLC treatment to be present on the cell surface, it was also shown to be active. N-linked glycans were therefore concluded not to be essential for either targeting of GP63 to the cell surface, or its activity. Mutational studies altering each of the three N-glycosylation sites, either individually or as a group, also suggested that loss of N-glycosylation did not prevent either surface expression of GP63 or its activity (McGwire and Chang, 1996). Tunicamycin treatment has also been examined in relation to the secreted protein acid phosphatase in *L. donovani* promastigotes. While the inhibitor affected the size of the protein it did not affect its secretion from the cell (Bates and Dwyer, 1987). However, the activity of the acid phosphatase was reduced (Lovelace and Gottlieb, 1987).

1.3.4 Function.

GP63 is classified as a protein with neutral metalloproteinase activity for a range of substrates. It is suggested that GP63, expressed abundantly in promastigotes, provides protection from hydrolytic enzymes in the insect gut. (Alexander *et al.*, 1999). In metacyclic promastigotes GP63 expression is upregulated and is thought to contribute to the survival of the parasite in the vertebrate bloodstream by possible inhibition of complement mediated lysis, and increasing opsonisation (Brittingham and Mosser, 1996). It has also been shown that expression of GP63 on the surface of mammalian cells converts those cells into efficient activators of complement, and the complement is opsonic and enhances binding to the complement receptor CR1 of the macrophage complement receptor system (Brittingham *et al.*, 1995).

Deletion of six of the seven *L. major* GP63 genes (genes 1-6) resulted in a mutant cell line with increased sensitivity to complement mediated lysis (Joshi *et al.*, 1998). A later study examined a strain where all seven *L. major* GP63 genes had been deleted, and compared this with WT *L. major*, and a deletion strain re-expressing GP63 gene-1 from a plasmid (Joshi *et al.*, 2002). Within the sandfly vector there was no phenotypic differences between the three strains. The GP63 deletion mutant was more sensitive to complement mediated lysis than WT cells, and whilst still able to infect mice, lesion development was significantly delayed in comparison to WT cells. Once the disease had been established the rate of lesion progression for WT and GP63 mutant strains was similar. The GP63 Gene-1 re-expressing cell line had an intermediate phenotype, infectivity and complement insensitivity were not completely restored to WT levels, possibly due to inappropriate gene regulation from the plasmid (Joshi *et al.*, 2002). In a different study an *L. mexicana* mutant cell line, Δ*gpi8*, which lacks GPI-anchored GP63 from the cell surface, was able to infect macrophage and differentiate and replicate at levels comparable to WT cells. The cell line was also able to infect mice, although wild-type *L. mexicana* amastigotes produced larger lesions which formed more rapidly than those caused by the mutant parasites (Hilley *et al.*, 2000). Therefore, whilst GP63 plays an important role in pathogenesis, it does not appear to be essential (Hilley *et al.*, 2000; Joshi *et al.*, 2002).

1.4 GPI biosynthetic pathway

GPI protein anchors are synthesised within the endoplasmic reticulum by a series of additions of sugars and other molecules to phosphatidylinositol (PI), the final step being the linkage of the complete anchor to a protein with a GPI-anchor attachment signal sequence. A core region of the GPI anchor is well conserved between higher and lower eukaryotes, but this is modified by different side chains in different organisms and cell types (McConville and Ferguson, 1993; Ferguson, 1999).

GPI biosynthesis was first characterised using a trypanosome cell free system. Introduction of radiolabelled substrates to permeabalised cells or membranes allowed the identification of GPI intermediates (Masterson *et al.*, 1989). Subsequently cell free systems were developed for a variety of mammalian cell types (Hirose *et al.*, 1992) and *Leishmania* (Smith *et al.*, 1997b). The highly conserved GPI backbone suggested that

the biosynthesis of GPI anchors was also highly conserved between species (Ferguson, 1999).

The genes involved in the GPI biosynthetic pathway have been most clearly defined in mammalian and yeast cells. The methods used for identifying the genes encoding the enzymes involved in the pathway include the generation and characterisation of panels of GPI deficient mutant cell lines (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991), complementation with cDNA (Inoue *et al.*, 1993), and the use of cell-free assay systems. A large number of GPI-deficient cell lines have been isolated and while GPI-anchored proteins are not essential in mammalian cells in culture, GPI-anchor deficient mice were embryonic lethal (Kawagoe *et al.*, 1996).

The GPI anchor is essential in yeast, possibly due to the requirement for the transportation and incorporation of GPI proteins into the cell wall (De Sampaïo *et al.*, 1999). Temperature sensitive mutants have been produced in which GPI anchoring is abolished when yeast cells are grown at 37°C (Leidich *et al.*, 1994; Benghezal *et al.*, 1995). Screening of mutagenized cells labelled with [^{3}H]inositol at 37°C allowed the identification of cell lines which were unable to incorporate [^{3}H]inositol. These cell lines were further analysed by incubation of washed membranes with a labelled substrate, and tested for the synthesis of GPI anchor intermediates to identify at which point in the GPI biosynthetic pathway the cells were defective. The genetic complementation of these cell lines allowed the isolation of the genes involved (Leidich *et al.*, 1994). GPI inhibitors, such as YW3548, which blocks the addition of ethanolamine phosphate (EtN-P) to the first mannose residue in yeast and mammalian cells, have also been used (Sütterlin *et al.*, 1998).

The genes involved in GPI biosynthesis have been less well characterised in protozoa. In *T. brucei* the production of viable GPI biosynthesis deletion mutants has proved difficult, suggesting an essential role for GPI-anchored proteins in *T. brucei*. However in a recent study a *T. brucei* GPI biosynthesis mutant, with a disruption in the gene *GPI10*, was produced. Procyclic cells were viable but only when cultured in non-adherent flasks (Nagamune *et al.*, 2000). In *L. mexicana* GPI biosynthesis mutants are viable (Hilley *et al.*, 2000). The GPI biosynthetic pathway is described with respect to

mammalian and yeast cells, and where known the *T. brucei* and *Leishmania* homologues are included. Figure 1.3 provides a summary of the mammalian GPI biosynthetic pathway, and a list of all homologues identified in mammalian, yeast and trypanosomatids is given in Table 1.1.

1.4.1 Step 1: Transfer of N-acetylglucosaminyl to phosphatidylinositol

The first step of the pathway is the transfer of N-acetylglucosaminyl (GlcNAc) from a donor, UDP-GlcNAc, to a phosophatidylinositol (PI) to form GlcNAc-PI (Masterson *et al.*, 1989). The enzyme involved in this reaction is GPI-GlcNAc transferase, and in mammalian cells this enzyme is a complex of at least 5 proteins, PIG-A, PIG-C, PIG-H, GPI1 and PIG-P (PIG refers to phosphatidylinositol glycan) (Watanabe *et al.*, 2000).

Assessment of a panel of mammalian cell lines (murine T-cell lymphoma cells), in which the GPI-anchored protein Thy-1 was synthesized but not expressed on the cell surface, identified 3 complementation classes (A, C and H), which were unable to transfer GlcNAc to the PI acceptor (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991). The human PIG-A gene was cloned by complementation of mammalian class A cell line (Miyata *et al.*, 1993). The protein has a small lumenal domain thought to be required to target the protein to the ER, and a large cytoplasmic domain (Watanabe *et al.*, 1996). Mutation of this gene is responsible for the disease paroxysmal nocturnal hemoglobinuria, characterised by abnormal blood cell populations due to a deficiency in complement regulating GPI-anchored molecules (Takeda *et al.*, 1993).

The yeast homologue of PIG-A, GPI3 was identified in *S. cerevisiae* by complementation of a temperature sensitive mutant, defective in the synthesis of GlcNAc-PI (Leidich *et al.*, 1995; Vossen *et al.*, 1995). Both PIG-A and GPI3 have homology to bacterial GlcNAc transferases (Kawagoe *et al.*, 1994; Vossen *et al.*, 1995), and photo-crosslinking experiments demonstrated that GPI3 could be directly crosslinked to a UDP-GlcNAc analogue (Kostova *et al.*, 2000). GPI3 and PIG-A are thought to be the catalytic components in the yeast and human forms of the GPI-GlcNAc transferase.

The gene encoding human PIG-H was cloned by complementation of the H class mutant cell line deficient in the ability to synthesize the first intermediate in the GPI

pathway (Kamitani *et al.*, 1993). PIG A and PIG-H directly associate with each other as demonstrated by co-precipitation experiments, though neither is required to target the other to the ER (Watanabe *et al.*, 1996). The ORF *YNL038w* of *S. cerevisiae* was identified as encoding the yeast homologue of human PIG-H, and was designated *GPI15* (Yan *et al.*, 2001). Disruption of the gene was lethal, however a temperature sensitive mutant cell line showed that the formation of [^{14}C]GlcNAc-PI in membranes from cells depleted of GPI15 expression was less than in membranes from cells expressing GPI15. The formation of GPI intermediates in whole cells was also shown to decrease when GPI15 expression was depleted. This demonstrated that GPI15 was a component of the yeast GPI-GlcNAc transferase (Yan *et al.*, 2001).

GPI1 was identified in *S. cerevisiae* by the production, isolation and analysis of a temperature sensitive mutant cell line (Leidich *et al.*, 1994), and complementation with a *S. cerevisiae* genomic library (Leidich and Orlean, 1996). Membranes from the temperature sensitive cell line grown at 37°C showed decreased ability to convert UDP[^{14}C]GlcNAc to [^{14}C]GlcNAc-PI, showing the defect was at the first step of the GPI biosynthetic pathway (Leidich *et al.*, 1994). Production of a Δ*gpi1* haploid cell line, in which a large portion of the *GPI1* gene was deleted, was still viable at 25°C, though it was non-viable at 37°C. At 25°C ^{3}H inositol was still incorporated into GPI-anchored proteins. This suggested that GPI1 was a non-essential protein within the GPI-GlcNAc transferase enzyme complex (Leidich and Orlean, 1996).

The mammalian *hGPI1* gene was identified by homology to the yeast *GPI1* (Watanabe *et al.*, 1998; Tiede *et al.*, 1998). Co-precipitation of a tagged form of GPI1 with each of the proteins PIG-A, PIG-H, and PIG-C demonstrated that in mammalian cells GPI1 directly interacts with each of these proteins. PIG-A, PIG-H, PIG-C and GPI1 could be co-precipitated together, suggesting that the four proteins interact as a complex (Watanabe *et al.*, 1998). GPI1 was disrupted in a mouse cell line, and in these cells PIG-A and PIG-H were easily detected as a complex, but a complex of PIG-A, PIG-H, and PIG-C was not detected (Hong *et al.*, 1999a). GPI1 is thought to be a membrane protein with 4 membrane spanning domains (Leidich and Orlean, 1996; Watanabe *et al.*, 1998). It is thought that GPI1 stabilises the GlcNAc-transferase enzyme complex linking PIG-A and PIG-H with PIG-C (Hong *et al.*, 1999a).

The gene encoding the *S. cerevisiae* GPI2 protein was isolated by complementation of a temperature sensitive mutant cell line using a yeast genomic library (Leidich *et al.*, 1995). The mutant cell line was shown to be defective in the first step of the GPI biosynthetic pathway, as membranes from cells grown at 37°C were unable to convert UDP[^{14}C]GlcNAc to [^{14}C]GlcNAc-PI. Analysis of the predicted protein sequence encoded by GPI2 suggested that it was a membrane protein with at least 2 potential membrane spanning domains (Leidich and Orlean, 1996). The gene encoding the human PIG-C gene was isolated by complementation of the C class mutant cell line (Inoue *et al.*, 1996). PIG-C is an ER membrane protein with 20% identity to the yeast GPI2 protein (Inoue *et al.*, 1996).

The human GlcNAC transferase complex was isolated by affinity purification from cells expressing tagged forms of PIG-A. SDS-PAGE revealed the complex was made up of 6 different proteins, one of which was revealed by N-terminal sequencing of the protein to be novel (Watanabe *et al.*, 2000). The cDNA for this protein, termed PIG-P was cloned, and used to complement a cell line deficient in GlcNAc transferase activity, such that GPI-anchored proteins were restored to the cell surface. (Watanabe *et al.*, 2000). A tagged form of PIG-P could be used to co-precipitate PIG-A and GPI-1, but not PIG-H or PIG-C when co-expressed. These experiments demonstrated that PIG-P was an essential component of the GlcNAc Transferase complex, and directly associated with PIG-A and GPI-1 (Watanabe *et al.*, 2000). Analysis of the predicted protein sequence of PIG-P suggests that it has 2 membrane domains, however it has no homology to other proteins with specific functions. Database analysis suggested that homologues of PIG-P also exist in mice, and *S. cerevisiae* (Watanabe *et al.*, 2000).

Analysis of the GlcNAc transferase complex from human cell lines also identified the protein DPM2, which is a component of the dolichol-phosphate-mannose (Dol-P-Man) synthase (Watanabe *et al.*, 2000). Dol-P-Man synthase is made up of three components DPM1, DPM2, and DPM3, however analysis of the GlcNAc transferase complex by western blotting or co-precipitation demonstrated that DPM2 but not DPM1 or DPM3 associates with GlcNAc Transferase (Watanabe *et al.*, 2000). Co-expression of DPM2 individually with each of the GlcNAc transferase complex members, followed by co-precipitation showed that DPM2 interacts with the GlcNAc transferase by direct

association with PIG-A, PIG-C and GPI1 (Watanabe *et al.*, 2000). The CHO Lec15 cell line, which was defective in *DPM2* (Maeda *et al.*, 1998), was analysed for GlcNAc transferase activity, and compared with the cell line re-expressing DPM2. Microsomes from both cell lines had GlcNAc transferase activity, as assessed by the conversion of UDP-GlcNAc to GlcNAc-PI and GlcN-PI, however in the mutant cell line re-expressing DPM2 the efficiency of this conversion was three-fold higher. DPM2 is not an essential component of the GlcNAc transferase, however it does appear to enhance activity and it was speculated that DPM2 may regulate GlcNAc transferase activity (Watanabe *et al.*, 2000).

1.4.2 Step 2: Deacetylation of GlcNAc-PI

The second step of the pathway is the deacetylation of GlcNAc-PI to form glucosaminyl-PI (GlcN-PI) catalysed by GlcNAc-PI deacetylase (Doering *et al.*, 1989; Milne *et al.*, 1994). Chinese hamster ovary (CHO) cells were mutagenised and a cell line was identified which did not express GPI-anchored proteins on the cell surface. Membranes from this cell line converted UDP-[6-^{3}H]GlcNAc to GlcNAc-PI, but not GlcN-PI demonstrating that the cell line was defective in GlcNAc-PI deacetylation. A gene, termed *PIG-L*, was identified by complemetation of the mutant cell line using a rat cDNA library. Analysis of the predicted protein sequence suggests that the protein has an amino terminal hydrophobic domain thought to attach the protein to the ER membrane (Nakamura *et al.*, 1997).

A yeast homologue, *GPI12*, was identified based on sequence homology to the mammalian gene *PIG-L* (Watanabe *et al.*, 1999). Expression of the *S. cerevisiae* GPI12 protein in the class L mutant CHO cells was able to restore GPI anchoring to these cells, demonstrating that GPI12 is the functional homologue of mammalian PIG-L. Attempts to produce an *S. cerevisiae* Δ*gpi12* cell line were unsuccessful suggesting that *GPI12* is an essential gene in yeast (Watanabe *et al.*, 1999).

The *T. brucei* GlcNAc-PI deacetylase was partially purified and characterised using a series of substrate analogues (Milne *et al.*, 1994). *T. brucei* and *L. major GPI12* homologues were cloned through homology to the *S. cerevisiae* and mammalian sequences, and each was shown to restore GPI anchoring when expressed in the class L mutant CHO cells (Chang *et al.*, 2002). Attempts to produce a *T. brucei* Δ*gpi12* mutant

cell line were unsuccessful suggesting GPI12 is an essential protein in *T. brucei*. However a *TbGPI12* conditional mutant was produced in which *TbGPI12* expression was controlled by induction with tetracycline. Membranes from the inducible cell line grown without tetracycline for 4 hours converted UDP[^{3}H]GlcNAc to [^{3}H]GlcNAc-PI, but showed a considerably lower conversion to the GPI intermediate [^{3}H]GlcN-PI compared to WT membranes. This demonstrated that *TbGPI12* encoded for a protein involved in GlcNAc-PI deacetylation (Chang *et al.*, 2002). Comparison of the human and trypanosome enzymes show some differences in substrate specificity, suggesting this enzyme is a potential drug target (Sharma *et al.*, 1997; Sharma *et al.*, 1999a).

1.4.3 Step 3: Inositol acylation

The third step in the mammalian pathway is inositol acylation, where a fatty acid is added to the inositol residue. This occurs prior to mannose addition in both mammalian cells and yeast. Cell lines deficient in the mannose donor mannose-P-dolichol (MPD) accumulate GlcN-acyl-PI (Costello and Orlean, 1992; Urakaze *et al.*, 1992). Use of synthetic analogues demonstrated that acylation was not obligatory for mannose addition to occur, however acylation increased the efficiency of mannosylation (Doerrler *et al.*, 1996). The genes encoding the GlcN-PI acyl transferase have not been identified. Acylation of the inositol confers PI-specific phospholipase C (PI-PLC) resistance to the GPI intermediate (PI-PLC is an enzyme which cleaves the phosphate lipid bond between the lipid component and inositol of GPI anchors).

The GPI biosynthetic pathway of *T. brucei* varies at this point from the mammalian pathway, as there is no requirement for the acylation of GlcN-PI for mannosylation to occur (Smith *et al.*, 1997a). Inositol acylation occurs only after the addition of the first mannose to GlcN-PI. All subsequent GPI intermediates exist as both inositol acylated and non-acylated forms. It is suggested that the inositol acylation of *T. brucei* GPI intermediates is required for the addition of ethanolamine, whilst inositol deacylation is required for fatty acid remodelling (Güther and Ferguson, 1995; Smith *et al.*, 1997a). A GPI inositol deacylase (*GPIdeAc*) has recently been cloned from *T. brucei* by exploiting the enzymes sensitivity to the inhibitor DFP, and using [^{3}H]DFP to identify the enzyme from trypanosome membranes (Güther *et al.*, 2001). The protein was predicted to be an ER lumenal glycoprotein. A *GPIdeAc* null mutant was produced.

The cells retained inositol deacylase activity, though at a reduced level, and the cells also showed an accumulation of inositol acylated GPI intermediates. This suggested the protein identified was an inositol deacylase but that other inositol deacylases were also present in the cells (Güther *et al.*, 2001). GPI intermediates from *Leishmania* are not inositol acylated (Smith *et al.*, 1997a).

1.4.4 Step 4: Mannose addition by mannosyltransferase I (MTI).

The three mannoses in the GPI core are linked to the backbone by different bonds, and therefore addition of each of the three mannose residues, contributed by dolichol phosphate-mannose (Dol-P-Man), is in three independent steps catalysed by different Dol-P-Man-dependent mannosyltransferases GPI-MTI, GPI-MTII and GPI-MTIII. Addition of the first mannose to the backbone forms Man-GlcN-acyl-PI. The substrate specificity of this step in the pathway differs between mammalian cells, trypanosomes and *Leishmania* and this is attributed to the varying acylation of the inositol ring (Smith *et al.*, 1997b).

A mammalian cell line, termed class M, was generated, defective in the addition of the first mannose to the GPI backbone, as demonstrated by the accumulation of the GlcN-acyl-PI intermediate (Maeda *et al.*, 2001). Complementation of the cell line with a rat cDNA library identified the mammalian *PIG-M* gene. Tagged forms of PIG-M were purified, and Man-GlcN-acyl-PI was generated when GlcN-acyl-PI and Dol-P-Man were incubated with the purified protein, indicating PIG-M had GPI-MTI activity. Analysis of the predicted protein sequence suggests that PIG-M may have up to 10 transmembrane domains. PIG-M also had a functionally important DXD motif, predicted to be a binding domain, positioned between the first and second transmembrane domains. DXD was predicted to be on the opposite side of the ER membrane from the N-terminal hydrophillic region. Tagging of the N-terminal of PIG-M and subsequent immunofluorescence microscopy demonstrated that the N-terminus was accessible to antibodies after permeabilization of the plasma membrane, and did not require the additional permeabilization of the ER membrane. The N-terminus was present on the cytoplasmic side of the ER membrane, and the functional domain of PIG-M was predicted to be in the ER lumen (Maeda *et al.*, 2001). *S. cerevisiae*, human

and *T. brucei* homologues were cloned through homology to the predicted rat PIG-M protein sequence (Maeda *et al.*, 2001).

Tagged forms of PIG-A, PIG-H and PIG-L have been demonstrated by microscopy and cell fractionation to localise to the ER (Watanabe *et al.*, 1996; Nakamura *et al.*, 1997). Microsomes prepared from the PIG-A, PIG-H and PIG-L mammalian mutant cell lines complemented with tagged forms of PIG-A, PIG-H, or PIG-L were exposed to Proteinase K digestion. An endogenous ER lumen protein, protein-disulfide isomerase (PDI) was protected from digestion, whilst PIG-A, PIG-H and PIG-L were not, suggesting a location on the cytosolic face of the ER for these proteins (Watanabe *et al.*, 1996; Nakamura *et al.*, 1997). GlcNAc-PI and GlcN-PI were susceptible to the PI-PLC treatment of intact microsomes prepared from mammalian cells and trypanosomes, suggesting these GPI intermediates were also present on the cytosolic face of the ER (Vidugiriene and Menon, 1993; Vidugiriene and Menon, 1994).

The first and second steps of GPI biosynthesis occur on the cytosolic face of the ER, whilst mannosylation of the GPI backbone occurs in the lumen of the ER (Maeda *et al.*, 2001). Subsequent steps in the pathway also occur in the ER lumen (Takahashi *et al.*, 1996; Hong *et al.*, 1999b; Ohishi *et al.*, 2000). The GPI intermediate is flipped from the cytosolic to the lumenal face of the ER at an undefined point in the pathway (Nakamura *et al.*, 1997). It is not clear if PIG-M, with its multiple transmembrane domains acts as the putative 'flippase' (Maeda *et al.*, 2001).

1.4.5 Step 5: Addition of ethanolamine phosphate to mannose

In mammalian, and yeast cells ethanolamine phosphate (EtN-P) is added to the first and second mannose residues of the GPI core. The modification of the mannose backbone by the addition of various side chains is one of the ways the conserved backbone of GPI anchors varies in different types of eukaryotes. Neither trypanosomes or *Leishmania* GPIs are modified with ethanolamine on the first and second mannose residues.

Yeast and mammalian GPI protein anchors are modified with EtN-P at position two of the first mannose. A yeast *mcd4* temperature sensitive mutant was shown to be defective in GPI anchoring (Gaynor *et al.*, 1999). MCD4 is homologous to a second yeast gene *GPI7*, responsible for the transfer of EtN-P to the second mannose

(Benachour *et al.*, 1999). Radiolabelling experiments on a temperature sensitive *gpi7* mutant cell line demonstrated that these cells accumulated a GPI anchor intermediate, termed M4, which lacked an EtN-P on the second mannose residue (Benachour *et al.*, 1999)

The mammalian gene *PIG-N* was cloned by homology to the yeast gene MCD4 (Hong *et al.*, 1999b). A mutant cell line was produced which was demonstrated to be deficient in the enzyme responsible for the transfer of EtN-P to the first mannose residue of the GPI intermediate, as a synthetic substrate analogue, GlcN-PI(C8), could be converted to ManGlcN-acylPI(C8) but no further. This demonstrated that PIG-N is responsible for the modification of Man1 with EtN-P. The PIG-N knock out cell line was still able to synthesise GPI intermediates with three mannose residue, and with EtN-P additions to mannose 2 and 3. PIG-N is specific for the addition of EtN-P to mannose 1 only, but this modification is not essential for the biosynthesis of the GPI anchor, though there is a reduction in the level of GPI-anchored protein (Hong *et al.*, 1999b). Sequence analysis suggests a *GPI7* homologue is also present in humans (Benachour *et al.*, 1999).

1.4.6 Step 6: Transfer of mannose 2 and 3

Two further Mannose residues are transferred to the GPI core structure, the mannose transferase (GPI-MTII) responsible for the addition of the second mannose has not been identified. PIG-B, which is associated with the addition of the third mannose has been identified in mammalian cells (Takahashi *et al.*, 1996). The Human *PIG-B* gene was cloned by complementation of a mutant cell line which accumulated a GPI precursor (M2) containing only 2 of the 3 mannose residues. The protein was shown to be an ER transmembrane protein with a small cytoplasmic domain, which was not required for activity, and a large lumenal domain (Takahashi *et al.*, 1996). *GPI10* was identified in *S. cerevisiae* by homology to *PIG-B* (Sütterlin *et al.*, 1998). The gene was essential, however a conditional mutant was produced in which *GPI10* was expressed under the control of the GAL1/10 promoter. When *GPI10* expression was prevented, inositol was no longer incorporated into proteins and the GPI intermediate Man_2-GlcN(acyl)PI accumulated. GPI10 could be replaced by PIG-B demonstrating that PIG-B is the functional homologue of GPI10 (Sütterlin *et al.*, 1998).

GPI10 from *T. brucei* was cloned by homology to PIG-B, and was able to restore GPI anchoring in the mammalian PIG-B defective cell line, and rescue yeast *gpi10* mutants (Nagamune *et al.*, 2000). Production of a Δ*gpi10* cell line in bloodstream form trypanosomes was not possible suggesting that GPI10 is essential in these cells. In procyclic cells double knockouts were produced when the cells were grown in non-adherent flasks. It was speculated that loss of the GPI protein procyclin resulted in the cells having an abnormally adherant surface. Labelling of the Δ*gpi10* cell membranes with GDP-[^{3}H]mannose demonstrated that the cell line was not able to synthesise the complete GPI anchor precursor (PP1), and accumulated precursors containing only 2 mannose residues (M2). [^{3}H]EP procyclin could not be detected in cells labelled with [^{3}H]myrstic acid, which is incorporated into GPI anchors, and EP procyclin could not be detected on the surface of the Δ*gpi10* cells. This demonstrated that GPI biosynthesis was disrupted in this cell line at the point of the addition of the third mannose to the GPI intermediate (Nagamune *et al.*, 2000).

1.4.7 Step 7: Transfer of EtN-P to the final mannose

The next step in the GPI biosynthetic pathway is the addition of EtN-P to the third mannose residue to produce a fully formed GPI anchor. The mutant murine thymoma cell line of complementation class F, accumulated the GPI anchor intermediate M3, which is an immediate precursor of the mature anchor. Complementation of the class F mutant cell line identified the human gene *PIG-F* (Inoue *et al.*, 1993). A second gene *PIG-O* involved in the transfer of EtN-P to the third mannose residue was identified by homology to the yeast EtN-P transferases GPI7, MCD4 and YLL031c (GPI13) (Hong *et al.*, 2000). Tagged forms of PIG-O and PIG-F could be co-precipitated demonstrating that they directly associate. A PIG-O deletion cell line was produced, in which the surface expression of a GPI-anchored protein was drastically decreased but not eliminated. Labelling experiments also demonstrated that while the PIG-F and PIG-O deletion cell lines both accumulated a major GPI anchor intermediate predicted to contain three mannose residues, each cell line also accumulated a different set of minor intermediates (Hong *et al.*, 2000). The predicted protein sequence of PIG-F was hydrophobic and it is thought to be a membrane protein (Inoue *et al.*, 1993). It was suggested that PIG-F, is not a catalytic component of the enzyme, but interacts with PIG-O and a third component to form the active enzyme. PIG-F and the third

component still retain some EtN-P transferase activity in the PIG-O deletion cell line (Hong *et al.*, 2000).

Sequence analysis suggested that yeast has a homologue of PIG-F, (Hong *et al.*, 2000). The *S. cerevisiae* gene YLL031c was identified by homology to *MCD4* and *GPI7* as a potential EtN-P transferase. Production of deletion mutant was not possible suggesting the gene was essential, however a conditional mutant was produced in which YLL031c was expressed under the control of an inducible promoter (Flury *et al.*, 2000). A tagged version of the protein was shown to have an ER location, and the high level of N-glycosylation was interpreted as suggesting the protein had a large luminal domain, despite the apparent degradation of the tagged protein when microsomes were exposed to Proteinase-K. YLL031c depleted cells also showed a decrease in the maturation of the GPI-anchored protein Gas1p, a phenotype associated with loss of GPI anchor addition to the protein (Doering and Schekman, 1996). Microsomes from the YLL031c depleted cells cell line made less of the complete GPI anchor precursor compared to WT microsomes, and instead accumulated a lipid termed 031b which was characterised as containing four mannose residues, but lacked EtN-P on Man3. This suggested that the cells were unable to attach EtN-P to the third mannose, and were therefore unable to attach the anchor precursor to the protein (Flury *et al.*, 2000).

1.4.8 Step 8: Addition of the complete anchor to an awaiting protein.

GPI anchor addition mechanism

The final step of the GPI anchor biosynthetic pathway is the removal of the hydrophobic COOH terminal of a protein and replacement with a GPI anchor. The mechanism for this addition has been the subject of much study. The rapidity of this GPI-anchor addition mechanism following translation of a protein destined to be GPI-anchored was first demonstrated in two separate studies in *T. brucei* (Bangs *et al.*, 1985; Ferguson *et al.*, 1986). Following the pulse-labelling of cells with ^{35}S methionine for 2 minutes, VSG could be immune-precipitated with both a VSG or CRD antibody, which detects the carbohydrate epitope which remains on a protein subsequent to cleavage of the GPI anchor. This suggested that a GPI anchor had been transferred to the protein. ^{3}H-myristate was also shown to be rapidly incorporated into VSG during pulse-chase labelling experiments, and it was concluded from these data that the GPI

anchor was added to an awaiting protein within 2 minutes of the proteins translation (Bangs *et al.*, 1985; Ferguson *et al.*, 1986). The rapidity of this modification indicated that the added anchor was pre-formed and added in its entirety to the awaiting protein (Bangs *et al.*, 1985). Addition of exogenous GPIs to a cell free system in *T. brucei*, confirmed that pre-formed GPI anchors were added to endogenous protein acceptors. This occurred even when membranes were incubated with protein synthesis inhibitors, indicating that ongoing protein synthesis was not required for GPI anchor attachment (Mayor *et al.*, 1991). Anchor addition did not occur outwith a very narrow detergent concentration, suggesting that anchor addition required proper membrane integrity, and that the transamidase enzyme was membrane associated (Mayor *et al.*, 1991).

The mechanism by which a pre-formed GPI anchor is linked to an awaiting protein was determined by a range of studies, many of which utilized cell free systems (Mayor *et al.*, 1991; Kodukula *et al.*, 1991; Sharma *et al.*, 1999b). These cell-free systems utilised washed membranes with endogenous GPI anchors, anchor accepting proteins, and transamidase activity. Alternatively the systems allowed the addition of the different components of the anchor addition reaction, such as *in vitro* translated anchor addition proteins modified to act as reporter proteins for different steps of the reaction. These systems allowed close regulation of the GPI-anchor addition reaction, and also eliminated the requirement to purify the enzyme involved in the reaction, which at that time had not been identified.

The precise mechanism for GPI anchor addition was thought to proceed by either of two methods, a transamidation reaction, or a 2-step reaction involving protein cleavage and anchor additon (Ferguson and Williams, 1988). The transamidation mechanism involves the cleavage of the carboxy-terminal extension of the protein and formation of an amide bond with the ethanolamine of the GPI in a single reaction. Alternatively cleavage of the protein by a signal peptidase may occur, followed by addition of the anchor by a transferase. A *T. brucei* cell free system was used to demonstrate that the anchor addition reaction did not require an exogenous energy source (Mayor *et al.*, 1991). In the absence of ATP and GTP the GPI anchor was still transferred to VSG. The lack of an energy requirement for the transfer reaction was consistent with a transamidation mechanism (Mayor *et al.*, 1991).

A cell free system utilizing the rough microsomal membranes (RM) of mammalian cells was developed (Bailey *et al.*, 1989), and was able to process an *in vitro* translated reporter protein specially modified to allow examination of the GPI anchor addition mechanism (Kodukula *et al.*, 1991). The reporter protein was adapted from the mammalian GPI-anchored protein placental alkaline phosphatase (PLAP), and contained the ER signal peptide, and the COOH terminal GPI addition sequence. The protein, miniPLAP, was 60% smaller than PLAP and glycosylation sites were absent. The sequential processing of four isoforms of the protein could be monitored by size, or by antibodies raised against epitopes from the NH_2 terminal end, COOH terminal end, or site of GPI anchor addition. PreprominiPLAP was the unprocessed non-GPI-anchored form, prominiPLAP lacked the ER signal, and was not GPI-anchored. GPI-miniPLAP was the GPI-anchored form, and free miniPLAP was the processed form lacking the ER signal, COOH hydrophobic domain and GPI anchor. PreprominiPLAP was transcribed *in vitro*, and translated using rabbit reticulocyte lysate. RMs were then added to this lysate.

Use of the miniPLAP system demonstated that there was a requirement for ATP during maturation of the GPI-anchored protein (Amthauer *et al.*, 1992), this was contradictory to previous findings (Mayor *et al.*, 1991). Subsequent investigation indicated that ATP hydrolysis was required to dissociate the pro form of the protein from the molecular chaperone, BiP, prior to transamidation (Amthauer *et al.*, 1993). It was proposed that BiP facilitates proper folding of the protein, or stabilises the proprotein until recognition by the transamidase occurs (Amthauer *et al.*, 1993). ER derived microsomes depleted of soluble lumenal components, such as BiP, were unable to process preprominiPLAP to miniPLAP, however when the lumenal content was removed after translocation of the prominiPLAP processing did occur (Vidugiriene and Menon, 1995). An energy dependent chaperone-mediated maturation step occurs prior to the energy independent transamidation reaction (Amthauer *et al.*, 1993; Vidugiriene and Menon, 1995).

Growth of cells mammalian cells in Brefeldin A, which disrupts the trans-Golgi network, failed to prevent GPI-anchor addition (Amthauer *et al.*, 1993), whilst a yeast mutant with a block in the secretory pathway between the ER and Golgi still added GPI

anchors to awaiting proteins (Conzelmann *et al.*, 1988). These results, together with the use of ER derived micosomes to reconstitute transamidase activity (Vidugiriene and Menon, 1995), indicated an ER membrane associated location for the GPI: protein transamidase (GPIT) (Conzelmann *et al.*, 1988; Amthauer *et al.*, 1993; Vidugiriene and Menon, 1995).

Use of the miniPLAP system demonstrated that in a cell free environment both GPI linked miniPLAP, and cell free mini PLAP could be synthesized (Maxwell *et al.*, 1995a). The production of free miniPLAP, lacking both a GPI anchor, and the COOH terminal sequence, was incompatible with a transamidation reaction. However in a previous study utilizing microsomes derived from a cell line which was unable to produce GPI anchors, no free miniPLAP was seen, leading to the conclusion that GPI anchors were required for the cleavage of the proteins GPI signal *in vivo* (Kodukula *et al.*, 1992). This was consistent with transamidation. From both sets of data it was concluded that a transamidation reaction does occur, with a complex forming between the GPI anchor, transamidase and prominiPLAP. This complex leads to the formation of a highly reactive carbonyl group on the protein which is susceptible to nucleophillic attack. This nucleophile is usually the ethanolamine group on the GPI anchor, resulting in a GPI-anchored protein. However in the cell free system another abundant nucleophile, such as water, could interact with the protein-transamidase intermediate in a competing reaction, resulting in the formation of free miniPLAP (Maxwell *et al.*, 1995a). This model suggested that the introduction of stronger nucleophiles could replace GPIs within the cell free system (Maxwell *et al.*, 1995a). This hypothesis was proved with the demonstration that enzyme catalysed cleavage of the GPI signal peptide occurred in the presence of hydrazine and hydroxylamine, even in the absence of an energy source and in GPI-deficient cells (Maxwell *et al.*, 1995b; Ramalingam *et al.*, 1996). The products formed were presumed to be miniPLAP-hydrazide and miniPLAP-hydroxamate, however due to the low abundance of these products they were not characterized. This set of experiments provided convincing evidence that the enzyme involved in GPI anchor addition was a transamidase.

A cell free assay using washed membranes from *T. brucei* procyclic cells expressing VSG was used to further clarify the transamidation mechanism (Sharma *et al.*, 1999b).

Hydrazine was used to release membrane associated GPI-VSG, but not transmembrane VSG. This release occurred in the absence of GPI biosynthesis and in an early compartment of the secretory pathway. Pro-VSG was therefore the substrate for the hydrazine mediated release. A biotin-linked hydrazine demonstrated directly that biotin-hydrazine was incorporated into VSG, and this incorporation was at the C-terminal, as indicated by the lack of susceptibility of VSG-hydrazide to carboxypeptidases. Previous studies had not been able to show directly that hydrazine was incorporated at the C-terminus of pro-proteins (Maxwell *et al.*, 1995b). Hydrazine replaced the GPI moiety in the transamidation reaction. Sulfhydryl alkylating reagents were shown to inhibit the transamidation reaction, suggesting that the transamidase contains a catalytically important cysteine residue (Sharma *et al.*, 1999b).

A GPI: protein transamidase (GPIT) reaction mechanism was proposed (Figure 1.4 A). The carbonyl group at the site of GPI anchor attachment on the awaiting GPI-anchored protein, is activated by a sulfhydryl group in the transamidase. An enzyme–substrate complex forms, and the C-terminal signal sequence is cleaved from the protein. The complex undergoes nucleophilic attack by the free amine on the EtN-P residue on the GPI anchor, and this results in protein linked to the GPI anchor by an amide bond, and restoration of the active sulfhydryl site in the transamidase (Sharma *et al.*, 1999b).

GPIT complex members.

The genes encoding the members of the GPIT have been identified in yeast and mammalian cells, and the enzyme appears to be a complex of at least 4 members. In yeast these are termed *GAA1*, *GPI8*, *GPI16* and *GPI17*. GAA1 was identified by complementation of a temperature sensitive mutant cell line, which synthesised the complete GPI anchor precursor, but was unable to incorporate it onto the GPI-anchored protein Gas1p. The GAA1 protein was characterised as containing 6 membrane spanning domains (Hamburger *et al.*, 1995). The mammalian *GAA1* was cloned by homology to the yeast protein, and had 28% identity at the amino acid level (Hiroi *et al.*, 1998).

GPI8 was identified by the complementation of a second defective yeast cell line, and the gene encoded a protein with a predicted N-terminal ER signal sequence and a C-terminal transmembrane domain (Benghezal *et al.*, 1996). Treatment of microsomes

with Proteinase K and cellular fractionation demonstrated that the protein had an ER lumen location (Benghezal *et al.*, 1996). Studies on mammalian cells identified a human K562 cell line with a mutation in GPI anchoring (termed complementation class K) (Mohney *et al.*, 1994). A later study demonstrated, using a cell free system, that these cells were defective at the point of anchor addition to awaiting proteins (Chen *et al.*, 1996). The human GPI8 gene was cloned by homology to the yeast protein, and was able to restore GPI anchoring in the class K cells (Yu *et al.*, 1997). The GPI8 protein showed a significant homology to a family of plant endopeptidases, some of which have transamidase activity, and GPI8 was predicted to be the catalytic sub-unit of the GPIT complex (Benghezal *et al.*, 1996).

The yeast protein GPI16 was identified as a third member of the GPIT complex by co-immune-precipitation of the complex, and was predicted to be N-glycosylated with a single C-terminal transmembrane domain (Fraering *et al.*, 2001). A mammalian homologue, PIG-T, was identified by a similar method (Ohishi *et al.*, 2001). This study also isolated a fourth member of the mammalian GPIT complex, termed PIG-S, and cloned a yeast homologue termed *GPI17*.

The GPIT complex of trypanosomatids has been less well characterised. The gene encoding GPI8 from *L. mexicana* was identified through homology to the yeast GPI8 protein (Hilley *et al.*, 2000). A GPI8 deletion strain (Δ*gpi8*) was produced, demonstrating that the protein was not essential in *L. mexicana* promastigotes grown in culture. Immunofluorescence demonstrated that the GPI-anchored protein GP63 was lost from the cells surface, while re-expression of the GPI8 protein within the Δ*gpi8* cell line restored GP63. The Δ*gpi8* cells accumulated protein anchor precursors. A histidine tagged form of the *Leishmania* GPI8 protein was used in the trypanasome cell free system and was able to restore GPI anchoring, after GPIT activity had been removed by a high pH wash (Sharma *et al.*, 2000). A *T. brucei* GPI8 homologue has also recently been identified (Kang *et al.*, 2002; Lillico *et al.*, 2003). *L. mexicana* and *T. brucei* GPI8 have no transmembrane domain as predicted from the gene sequences, and appear to be soluble homologues of the yeast and mammalian enzymes (Hilley *et al.*, 2000; Kang *et al.*, 2002; Lillico *et al.*, 2003)

Recent studies have attempted to define the function of each of the identified complex members within the GPIT enzyme. The role of GPI8 as the catalytic sub-unit of the enzyme has been characterised in some detail. These results are described more fully, and discussed in relation to the situation in *Leishmania* and the trypanosomatids in section 3.5.

1.4.9 The GPI-anchor addition site

Proteins destined to be GPI-anchored show little sequence homology at the C-terminal end, however all proteins possess 2 common features; a GPI attachment signal, and a hydrophobic domain at the C-terminus (Ferguson and Williams, 1988) (Figure 1.4 B). The hydrophobic domain has been shown to be necessary for anchor attachment. A model system studied the mammalian GPI-anchored protein decay accelerating factor (DAF), transiently expressed from a vector in COS (CV1 origin-deficient SV-40) cells (Caras *et al.*, 1989). Deletion or shortening of the 17 amino acid hydrophobic domain prevented GPI-anchor addition to DAF, whilst fusion of this domain to a secreted protein did not result in anchor addition (Caras *et al.*, 1989). Replacement of the 17 amino acid domain with a random hydrophobic sequence, or attachment of the non-anchored protein hGH (human growth factor) at the C-terminal end of DAF, such that the hydrophobic domain and associated attachment sequence were positioned in the middle of a large hydrophilic fusion protein, both resulted in the efficient GPI anchoring of DAF (Caras and Weddell, 1989; Caras, 1991). It was concluded from these experiments that the C-terminal hydrophobic domain was essential but not sufficient for anchor addition, and that the function of the domain was not due to the precise sequence, but its hydrophobic properties.

The amino acid at the site of GPI anchor attachment has undergone much study, and has been termed the ω residue (Gerber *et al.*, 1992). The GPI anchor is attached to the protein on the C-terminal side of the ω residue. Site directed mutagenesis studies showed that the only requirement for the ω residue was that it had a small side chain (Asparagine, serine, glycine, alanine, aspartic acid, and cystiene). (Micanovic *et al.*, 1990; Moran *et al.*, 1991; Nuoffer *et al.*, 1993).

The ω site of the human protein Decay accelerating factor (DAF) was identified as serine^{319}, 12 residues N-terminal to the C-terminal hydrophobic domain. Attachment of the DAF hydrophobic domain to the C-terminal end of the non-GPI-anchored receptor protein LDL was insufficient to produce a GPI-anchored fusion protein. Subsequent addition of both a serine and glycine residue 12 or 9 amino acids N-terminal to the hydrophobic domain did produce a GPI-anchored protein. Addition of only a serine residue, or addition of the serine and glycine residues at other positions failed to produce a GPI-anchored protein (Moran and Caras, 1991). This suggested that the only requirement for GPI anchor addition was an anchor addition site consisting of a pair of small amino acid residues positioned 9-12 residues N-terminal of a hydrophobic domain.

Site directed mutagenesis studies using preprominiPLAP expressed in a cell free system (Kodukula *et al.*, 1991) examined the residues C-terminal to the ω residue (ω+1 and ω+2) (Gerber *et al.*, 1992). GPI-anchor addition occurred when the ω+1 site was any residue with the exception of proline. The ω+2 site was more limited, in that anchor addition only occurred when alanine or glycine were in this position, a small amount of processing also occurred with serine (Gerber *et al.*, 1992). These results were repeated when WT PLAP was expressed in intact cells. The addition of threonine or valine at the ω+2 site also resulted in some processing but at a reduced level (Kodukula *et al.*, 1993). It was suggested that the ω, and ω+2 residues were highly predictive of the point of GPI anchor addition (Kodukula *et al.*, 1993). These results appeared to contradict the findings from the DAF system, whereby only 2 small residues were required at the ω, and ω+1 sites (Moran and Caras, 1991). A study on the *S. cerevisiae* GPI-anchored protein Gas1p also indicated that the ω and ω+2 site were important in determining GPI anchor addition, however it was also suggested that the residues tolerated at each position (ω, ω+1, and ω+2) were dependent on which residues were present at the other two sites (Nuoffer *et al.*, 1993). A predictive model was developed based on the ω and ω+2 sites (Udenfriend and Kodukula, 1995), whilst a later model was developed using a computer based analysis of GPI-anchored proteins, which identified conserved sequence properties from ω-11 to ω+5 (Eisenhaber *et al.*, 1998).

Analysis of miniPLAP and a fusion protein consisting of the mature domain of miniPLAP fused to the C-terminus of the GPI-anchored protein the urokinase receptor, allowed a direct comparison of mutations on different GPI anchor signals (Aceto *et al.*, 1999). This demonstrated that the requirements for the anchor addition domain in different proteins is highly variable, influenced partly by the residue at the ω+4 position. In some cases a second cleavage site was utilized (Aceto *et al.*, 1999).

Sequence analysis of GPI-anchored proteins suggest that some differences exist between the mammalian and protozoan GPI attachment site. Protozoa favour different amino acids at the ω and ω+2 site compared to mammalian proteins. The expression of VSG (variant 117) in COS cells resulted in inefficient GPI anchoring, as assessed by surface expression and ethanolamine labeling (Moran and Caras, 1994). The production and expression of VSG-DAF fusion proteins in COS cells suggested that this failure was due to the VSG GPI signal functioning poorly in mammalian cells. Addition of the DAF ω site and C-terminal end to VSG or human Growth Hormone (hGH) using this expression system resulted in GPI-anchored protein, whilst addition of the VSG ω site and C-terminal to hGH did not (Moran and Caras, 1994). Replacement of the DAF C-terminal hydrophobic domain with that of VSG resulted in the production of GPI-anchored DAF, indicating the hydrophobic domain did not influence differences in GPI-anchoring between mammalian and human cells. It was suggested that the defect in anchor addition was due to the different requirements at the anchor addition site between protozoan and mammalian cells (Moran and Caras, 1994). However a later study converted the predicted GPI-anchor attachment site of porcine membrane dipeptidase (MDP) to that found in VSG (variant 117) and expressed the protein in COS cells (White *et al.*, 2000). The mutated form of MDP was GPI-anchored on the cell surface, suggesting that the VSG anchor addition site was viable in the mammalian system, and that the requirements at the anchor addition site between mammalian and protozoan cells were not as variable as suggested (White *et al.*, 2000).

An extensive study mutating the ω, ω+2, ω+3, ω+4, ω+5, ω+7 and ω+8 positions of VSG 117 by site directed mutagenesis, and analysis of the mutated proteins by expression in *T. brucei* did not prevent GPI-anchor addition (Bohme and Cross, 2002). This suggested that the GPI-anchor addition site of VSG was not as conserved as had

been previously predicted, though it was also considered possible that after mutation an alternate addition site was utilised (Bohme and Cross, 2002). The variability in anchor addition requirements between individual proteins (Moran and Caras, 1994; Aceto *et al.*, 1999; White *et al.*, 2000), and the ability to utilise secondary anchor addition sites in the event of mutation (Aceto *et al.*, 1999), suggested that anchor addition requirements are extremely complex and may be specific for the particular protein studied (White *et al.*, 2000).

1.5 Processing and trafficking of GPI-anchored proteins

The processing and trafficking of GPI-anchored proteins to the cell surface in higher eukaryotes is thought to follow a broadly defined, highly organised pathway. Proteins are trafficked into the ER where differentiated domains exist for processes such as protein folding, lipid metabolism, and membrane transport, and are then transported to the Golgi body, for further processing. The mature protein enters the trans-Golgi network (TGN), where it is sorted and packaged for export to the cell membrane or secretion (Lippincott-Schwartz *et al.*, 2000). Secretory transport in trypanosomatids is less well defined, but is thought to follow the general pathway found in higher eukaryotes, though all endocytosis and exocytosis to the cell surface occurs via the flagellar pocket (Clayton *et al.*, 1995; Overath *et al.*, 1997; McConville *et al.*, 2002b).

Ultrastructural studies of high pressure frozen *L. mexicana* cells show the intracellular distribution of GPI-anchored proteins and give a detailed picture of the intracellular architecture of these cells (Weise *et al.*, 2000). Organelles involved in the trafficking of secreted molecules are clustered at the anterior end of the cell close to the flagellar pocket. An ER region with an intense budding pattern is located close to the cisternal face of the Golgi, and these budding vesicles appear to form the new cisternae on the cis-face of the Golgi apparatus (Weise *et al.*, 2000). The area between the ER and Golgi appears less complicated than that found in mammalian cells, with no regions which could be interpreted as an ER-Golgi intermediate compartment (ERGIC) found in mammalian cells (Weise *et al.*, 2000). Flattened vesicles bud from the trans-face of the Golgi, and these can be equated with the trans-Golgi network (TGN) present in mammalian cells. The flattened vesicles appear to be in transit between the Golgi, and large translucent vesicles, which in turn appear to connect with the flagellar pocket and

tubular clusters. These large vesicles contained biotinylated material and it was speculated that these structures are involved in the sorting and recycling of endosomes, similar to the system found in mammalian cells (Weise *et al.*, 2000).

The enzyme dolichol-phosphate mannose synthase (DPMS), catalyses the synthesis of dolichol phosphate mannose, a mannose donor required for GPI biosynthesis, on the cytosolic face of the ER (Ilgoutz *et al.*, 1999b). Production of a functionally active GFP tagged form of this enzyme was produced in order to identify sub-compartments of the ER important in GPI, LPG, and GIPL biosynthesis (Ilgoutz *et al.*, 1999a). The chimera localised to a stable tubular compartment, which extended from the flagellar pocket toward the posterior end of the cell (Ilgoutz *et al.*, 1999a). This multivesicular tubule (MVT) was also identified by electron microscopy and was flanked by microtubules and contained a variety of different sized vesicles (Weise *et al.*, 2000). Labelling of the MVT with surface biotinylated GP63, and antibodies directed against phosphoglycan structures which are formed post ER suggested that the structure was not an ER compartment but was a post-Golgi compartment (Weise *et al.*, 2000). Subcellular fractionation suggested that the MVT may contain cysteine proteases, and it was also observed that the MVT was labelled with the endocytic marker FM 4-64, but that this compound was not chased any further (Mullin *et al.*, 2001). This suggested that the MVT is a late lysosomal compartment. (Weise *et al.*, 2000; Mullin *et al.*, 2001). Disruption of the MVT with the inhibitor Balfomycin (which specifically perturbs lysosome/ endosome function), did not effect the trafficking of GPI-anchored GP63 to the cell surface, and it was concluded that the MVT was not a compartment in the secretory pathway (Mullin *et al.*, 2001).

Ultrastructural studies suggest that the secretory pathway of the trypanosomatids is broadly similar to that found in mammalian cells. The processes involved in this pathway are briefly described in relation to mammalian cells, and related to the enzymes and pathways so far identified in trypanosomatids.

1.5.1 Translocation and ER Processing

Proteins destined to enter the secretory pathway have an N-terminal ER signal direction sequence. This signal binds to the signal receptor protein (SRP) during synthesis of the protein, forming a ribosome/ nascent protein/ SRP complex. The complex targets to the

SRP receptor on the membrane of the ER adjacent to the translocon. Transfer of secreted proteins from the ribosome into the ER occurs via the translocon. The translocon has an ER membrane spanning aqueous pore, which in mammalian cells has the core components of translocon associated membrane protein (TRAM), and a sec61 complex (Johnson and van Waes, 1999). Removal of the signal peptide by signal peptidase (SP), and N-glycosylation by the oligosaccharide transferase (OST) both occur as the protein is translocated into the ER lumen.

N-linked glycans are complex structures, the basic core structure of which is synthesised by a glycan biosynthetic pathway, this core is then added to the protein by the OST during translocation of the protein into the ER (Johnson and van Waes, 1999). Further modifications and additions to the glycan can occur as the glycosylated protein moves through the ER and Golgi apparatus (Dwek, 1996). The OST is considered to be an integral part of the translocon, though direct interaction between it and other translocon sub-units has not been demonstrated (Reviewed by Johnson and van Waes, 1999).

Little is known about the translocon in trypanosomatids. A mammalian signal sequence has been used to control the secretion of interferon-gamma in *L. major*. The protein was processed and secreted from the cells, suggesting that the translocation machinery is similar in both *Leishmania* and higher eukaryotes (Tobin and Wirth, 1993). VSG could not be imported into canine pancreas microsomes, unless its signal peptide was replaced with a yeast signal. GP63 was not imported into the microsomes, unless its signal peptide was altered by replacing 9 amino acids with those from *Autographa californica*. This suggests some diversity in the ER signal peptide exists between species (Al-Qahtani *et al.*, 1998).

The molecular chaperone, BiP, is a ubiquitous protein within the ER lumen of mammalian cells, and appears to have multiple functions. It is associated with the translocation of proteins across the ER membrane, assists in the folding of protein by reversibly binding to polypeptide chains, and may be responsible for blocking the translocon pore to prevent retrotranslocation (Reviewed in Johnson and van Waes, 1999). A homologue has been identified in *T. brucei*, the predicted protein sequence of which has 64% identity with rat BiP (Bangs *et al.*, 1993). The protein has an ER

retention signal, and immunofluorescence microscopy demonstrated that the protein localised to the ER. A BiP homologue has not yet been identified in *Leishmania*, however, the BiP antibody specific to *T. brucei* also identifies an ER located protein in *L. donovani* (Debrabant *et al.*, 2002).

In higher eukaryotes a variety of quality control mechanisms exists in the ER, which prevent the continued trafficking of misfolded proteins. ER associated degradation (ERAD) retrotranslocates misfolded proteins to the cytosol, where they are degraded in proteasomes. A build up of misfolded proteins in the ER triggers the unfolded protein response (UPR) (Ng *et al.*, 2000). The calnexin calreticulin pathway modulates the forward trafficking of glycoproteins (Parodi, 2000; Ellgaard and Helenius, 2001). N-glycosylation occurs as proteins are translocated to the ER lumen. The subsequent trimming of these N-glycans by Glucosidase I and II (GI and GII) allows the interaction of the glycosylated protein with the lectins calnexin or calreticulin. This association exposes the folding protein to ERp57 a molecular chaperone. The interaction is terminated by the removal of glucose from the glycan by GII and the protein is trafficked to the Golgi. If the protein is not in its correct conformation glucose is reattached to the protein by UDP-Glc:glycoprotein glucosyltransferase (GT), tagging it for reinteraction with the calnexin/ calreticulin cycle, and retaining the protein within the ER.

The mechanisms of quality control within the ER of trypanosomatids, are at present poorly defined. GII and GT activity has been detected in trypanosome cells (Parodi *et al.*, 1983; Bosch *et al.*, 1988; Trombetta *et al.*, 1989). Homologues of calreticulin have been identified in *L. donovani* and *T. cruzi*, the predicted proteins of which have the same characteristic domains identified in calreticulin homologues from higher eukaryotes (Joshi *et al.*, 1996; Labriola *et al.*, 1999). Over expression of the calreticulin P-domain in *L. donovani*, resulted in the reduction of secretion of acid phosphatase, and its intracellular retention (Debrabant *et al.*, 2002). This suggests that a similar quality control system to the calnexin/ calreticulin cycle of mammalian cells exists in trypanosomatids.

The ER of mammalian cells is an array of interconnecting membranes and cisternae organised into localised sub-domains specialised for different functions such as protein

folding, lipid metabolism and membrane transport (Lippincott-Schwartz *et al.*, 2000). Fractionation of thyoma cells demonstrated that some of the enzymes associated with GPI biosynthesis are confined to a sub-domain of the ER (Vidugiriene *et al.*, 1999). The trypanosomatid ER has been less well defined, though ultrastructural studies suggest that the ER of these organisms is similarly organised into distinct compartments (Weise *et al.*, 2000). The presence of distinct GPI biosynthetic pathways for the production of GIPL and GPI protein anchors and LPGs in *L. mexicana*, suggests that some compartmentalisation of the ER must occur (Ralton and McConville, 1998; Ilgoutz *et al.*, 1999b).

1.5.2 ER to Golgi transport

In mammalian cells the trafficking of secretory proteins from the ER to and through the Golgi is thought to occur by a generalised model (Lippincott-Schwartz *et al.*, 2000). Transport vesicles form in the ER, which traffic cargo proteins possibly via ERGIC whereby the vesicles fuse to form Golgi cisternae. ER/ Golgi intermediates were originally thought to be stable compartments (Lotti *et al.*, 1992), but it has since been suggested they are transient transport vehicles (Lippincott-Schwartz *et al.*, 2000).

In mammalian cells the small GTPase, Rab2, is associated with ER to Golgi transport, and the maturation of ER-Golgi intermediates (Tisdale and Balch, 1996). A homologue of Rab2 has been identified in *T. brucei* (Field *et al.*, 1999). Over-expression of TbRab2 in *T. brucei* resulted in a decrease in the level of procyclin synthesis, which is the major GPI-anchored surface protein of procyclic cells, and is thus the major cargo in the secretory pathway. Over-expression also resulted in the disruption of the ER by excess vacuolisation. Fluorescence microscopy suggested that TbRab2 co-localised with BiP, though in an incomplete fashion, whilst expression of TbRab2 in COS cells suggested the protein targeted to the ERGIC (Field *et al.*, 1999). It was suggested that ER/ Golgi intermediates may form in *T. brucei* (Field *et al.*, 1999). Electron microscopy on high pressure frozen cells was unable to identify complex ERGIC like structures in *Leishmania*, though the area of ER directly opposite the Golgi appeared to be highly specialised with cisternal like extensions and budding (Weise *et al.*, 2000). This specialised area of the ER was termed the transitional ER (tER).

1.5.3 Progression through the Golgi

The Golgi apparatus is organised as a series of stacks of cisternae, which appear to be polarised between the cis and trans faces. The method of transport through the Golgi is unclear but is thought to occur either by cisternal maturation, or by vesicular trafficking between stable compartments (Glick and Malhotra, 1998). Cargo proteins are transported through the Golgi to the TGN, here COPII/ clatherin coated vesicles form and continue anteriograde transport of the cargo proteins from the cell. Retrograde transport of resident Golgi proteins maintains polarity of the Golgi apparatus, and recycles proteins back to the ER (Glick and Malhotra, 1998). COPII (coat protein complex II) vesicles are involved in the anteriograde transport of proteins from the ER to the Golgi. The COPII coat consists of five proteins, the small GTPase Sar1p, and the heterodimers Sec23/24p and Sec13/31p. COPI vesicles are involved in retrograde transport whereby proteins are recycled from the cis-Golgi to ER. The COPI coat consists of a GTPase (Arf 1p and/ or ARF2p) and a 'coatmer' (consisting of seven sub units) (Springer *et al.*, 1999).

Brefeldin A (BFA) inhibits secretion by disruption of the Golgi body. It does this by inhibiting some of the proteins (GEFs) involved in the activation of the ADP-ribosylation factors (ARF) (Donaldson *et al.*, 1992). The ARF proteins are involved in the assembly of COPI vesicles responsible for the retrograde transport from the cis-Golgi to the ER (Roth, 1999). Inhibition of retrograde transport leads to the collapse of the Golgi and its redistribution into the ER. Yeast cells are insensitive to BFA due to the impermeability of the cells. However in permeable mutant strains BFA blocks secretion and affects Golgi morphology, by inhibition of the GEF proteins Gea1, Gea2 and Sec7 (Peyroche *et al.*, 1999). In mammalian cells BFA causes the collapse of the Golgi complex however only one of the mammalian GEFs identified is sensitive to BFA (Chardin and McCormick, 1999). Treatment of *T. cruzi* epimastigotes with BFA also disrupted the ultrastructure of the Golgi apparatus by causing an increase in the number and size of the cisternae (Engel *et al.*, 1998).

1.5.4 Trans-Golgi network to flagellar pocket

The TGN of mammalian cells is responsible for the sorting and packaging of transport intermediates. From the TGN proteins are trafficked to both the cell surface, and a

variety of compartments in the endosomal system. The complex mechanisms involved in the regulation of these pathways and sorting processes are at present only partially understood (Keller and Simons, 1997; Lippincott-Schwartz *et al.*, 2000). The trans-cisternae of the Golgi apparatus is speculated to be functionally equivalent to the TGN in trypanosomatids (McConville *et al.*, 2002b). Electron microscopy demonstrated that in *L. mexicana* this region undergoes the budding of different sized vesicles, and is closely associated with tubular clusters and larger vesicles (Weise *et al.*, 2000). The GRIP domain, which is a targeting domain specific for the mammalian TGN, was also identified on a *T. brucei* protein. This TbGRIP domain could be used to localise GFP to both the TGN of mammalian cells, and the trans-face of the Golgi apparatus in *L. mexicana* (McConville *et al.*, 2002a).

All endocytosis and exocytosis in trypanosomatids occurs via the flagellar pocket (Overath *et al.*, 1997). In *T. brucei* the density of VSG at the flagellar pocket was shown to be approximately 50 times higher than the density in the ER (Grunfelder *et al.*, 2002). Incubation of cells at 20°C inhibits intracellular transport between the trans-Golgi and cell surface. Transport of VSG to the surface of *T. brucei* cells was prevented by this treatment, and VSG was shown to have accumulated in the region of the trans-Golgi (Duszenko *et al.*, 1988). Immunogold labelling and electron microscopy demonstrated that *L. mexicana* GP63 was trafficked via large translucent vesicles, and tubule clusters, both of which appeared to associate directly with both the trans-Golgi, and flagellar pocket (Weise *et al.*, 2000).

1.5.5 GPI anchors and secretion

GPI anchors have been implicated in the correct forward transport of GPI-anchored proteins from the cell. Addition of a GPI anchor signal peptide to human growth hormone (hGH), which is normally secreted, resulted in the protein being targeted to the cell membrane. Mutation of the fusion proteins anchor addition site resulted in the uncleaved and unanchored protein accumulating intracellularly in a post-ER compartment (Moran and Caras, 1992), where it appeared to aggregate and was then degraded (Field *et al.*, 1994).

Inositol starvation of yeast cells prevented GPI-anchor modification of the protein Gas1p, and proper processing. The immature protein accumulated intracellularly

(Doering and Schekman, 1996). The yeast GPI-anchored protein Gas1p has been shown to associate with COPII vesicles in its transport from the ER, and loss of the GPI anchor inhibits this (Doering and Schekman, 1996). Mutation of Ret1p, a protein which is a coat component of COPII vesicles, also blocks GPI-protein transport from the ER (Sütterlin *et al.*, 1997). Retention of GPI-proteins in the ER may be due to the requirement of GPI anchor for forward transport, or instead, a result of the uncleaved GPI signal peptide acting as a strong retention signal.

Studies in *T. brucei* used a reporter system, based on a truncated form of VSG lacking the C-terminal GPI-signal peptide (VSGΔgpi), expressed in procyclic cells. This protein was exported from the cell with greatly reduced kinetics, compared with the rate wtVSG reached the cell surface. The procyclin GPI-signal peptide, restored anchor addition and transport efficiency (Bangs *et al.*, 1997). VSGΔgpi was shown to accumulate in the ER, and was demonstrated to be correctly folded and dimerised. The truncated protein lacked the hydrophobic C-terminal GPI-signal, previously suggested to be responsible for ER retention. It was suggested that the GPI anchor is necessary for the forward transport of some GPI-anchored proteins (McDowell *et al.*, 1998). In *Leishmania* GP63 mutated at the GPI-anchor addition site and expressed in a GP63 deficient cell line was demonstrated to be secreted from the cell, however the kinetics of secretion was not shown (McGwire and Chang, 1996).

1.6 Aims.

The extent of GPI-anchoring in the trypanosomatid protozoa demonstrates that this is an important process in these organisms. The production of a viable *L. mexicana* cell line deficient in GPI biosynthesis, suggest that *Leishmania* is an ideal organism in which to study GPI-anchor addition and biosynthesis. Recent research also indicates that there may be some significant differences between the GPIT complex in trypanosomatid protozoa and higher eukaryotes. The cloning of the GPI8 homologue from *L. mexicana*, and the generation of a Δ*gpi8* mutant cell line provided a useful tool for the study of GPI anchor addition in this organism. The *Δgpi8* cell line also provided a method of comparing the trafficking of GPI-anchored, and unanchored proteins through the cell, and assessing the significance of the GPI anchor in this process.

This study had 3 aims:

1. The characterisation of GPI8 and the GPIT complex by the determination of the active site residues of GPI8, the production of active site mutants and the examination of the role of GPI8 as part of a larger complex.

2. Characterisation of the trafficking of GPI-anchored proteins, and comparison with the situation in cells with a defect in GPI anchor addition.

3. Identification of novel genes associated with GPI anchor biosynthesis and trafficking, and the development of a novel screening method for the isolation of biosynthesis and trafficking mutants.

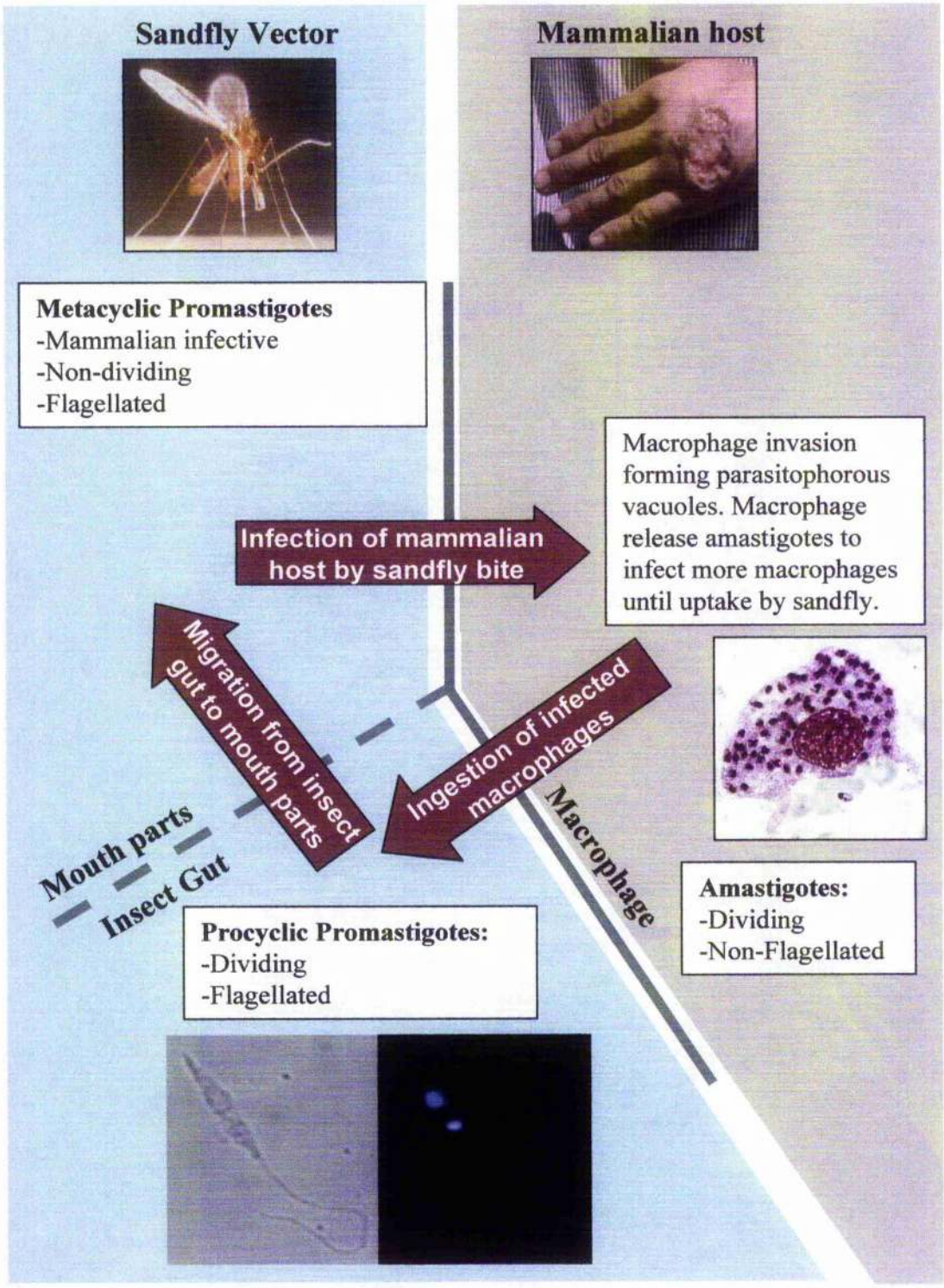


Figure 1.1: The lifecycle of *Leishmania*

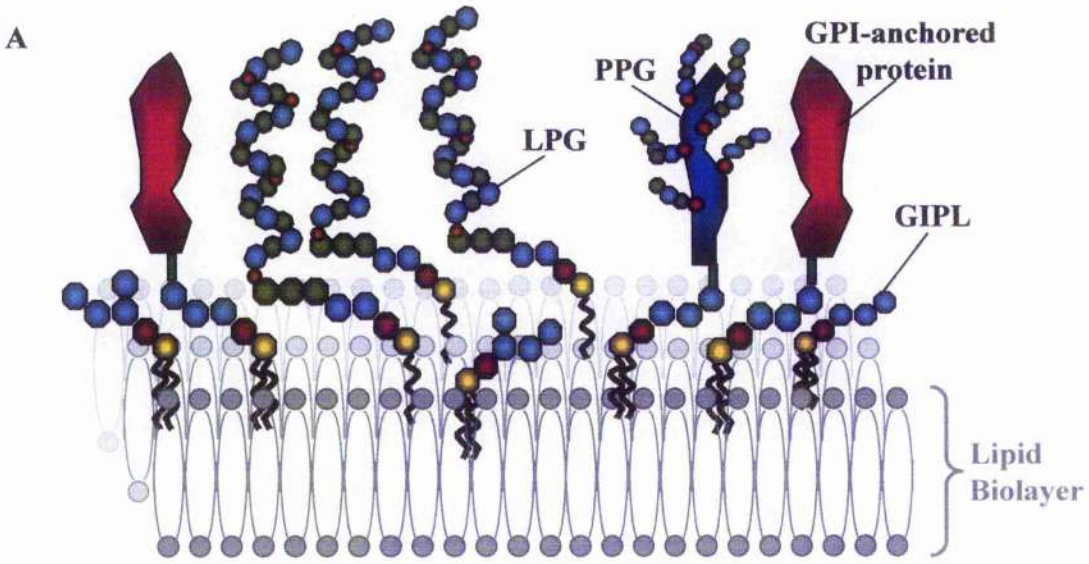
The lifecycle is described in detail in section 1.1.3. Images were taken from web site: http://www-medlib.med.utah.edu/parasitology/image.html

Figure 1.2: Structure of glycoconjugates in *Leishmania*

Panel A) Schematic of the glycoconjugates present on the surface of *Leishmania* promastigote cells.

Panel B) Structure of the GPI molecules present in Leishmania.

The GPI-anchor of GP63 is shown as an example of the common core structure present on GPI-anchored proteins. M3, GIPL-A, iM4, are shown as examples of Type 1, Type 2, and Hybrid Type GIPLs. The generic structure of the LPG anchor is shown.



B

GP63

GPI- protein anchor (GP63)

$NH-CH_2-CH_2$

PO_4

6

$Man\alpha1-2Man\alpha1-6Man\alpha1-4GlcNH_2\alpha1-6$ Ino

$PO_4-CH_2-CH-CH_2$

GIPLs

$Man\alpha1-2Man\alpha1-6Man\alpha1-4GlcNH_2\alpha1-6$ Ino

$PO_4-CH_2-CH-CH_2$

Type 1

$Man\alpha1-2Man\alpha1-6Man\alpha1-4GlcNH_2\alpha1-6$ Ino

3

$Man\alpha1$

$PO_4-CH_2-CH-CH_2$

Hybrid Type

Type 2

$Gal\beta1-3Gal\alpha1-3Gal\beta1-3Man\alpha1$ / $3Man\alpha1-4GlcNH_2\alpha1-6$ Ino

$PO_4-CH_2-CH-CH_2$

LPG

PO_4

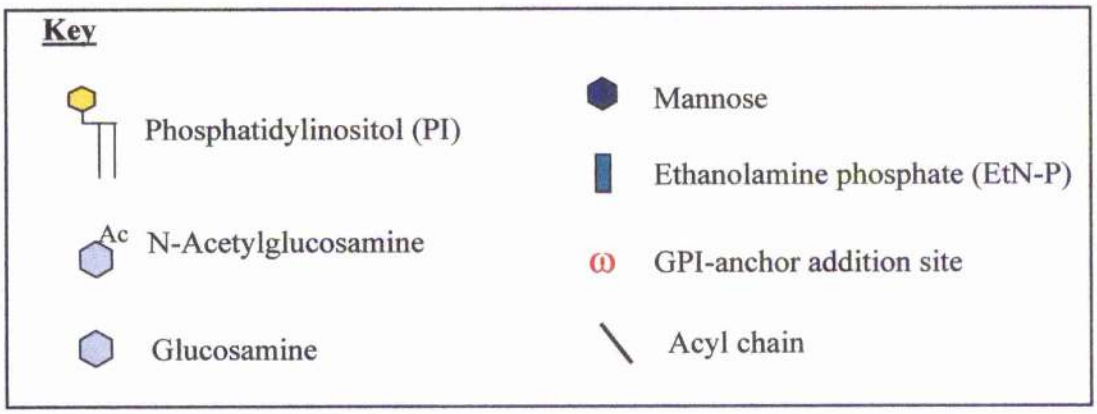
6

$Gal\beta1-6Gal\alpha1-3Gal\beta1-3Man\alpha1$ / $Glc\alpha1-PO_4$ (6) / $3Man\alpha1-4GlcNH_2\alpha1-6$ Ino

$PO_4-CH_2-CH-CH_2$

OH O

Figure 1.3: The GPI biosynthetic pathway of mammalian cells



Step 1:GlcNAc Transferase

Transfer of N-Acetylglucosaminyl (GlcNAc) from UDP-GlcNAc to a phosphatidylinositol (PI)

Step 2: GlcNAc-PI Deacetylase

GlcNAc-PI is N-deacetylated to form GlcN-PI

Step3: Inositol-Acyltransferase

An acyl chain is attached to the inositol ring.

Step4: Mannosyltransferase (GPI-MTI)

A mannose residue is transferred from dolichol phosphate mannose (dol-P-man) to the backbone.

Step5: EtN-P Transferase

Addition of EtN-P to the first and second mannose residues (higher eukaryotes only).

Step6: GPI-MTII and GPI-MTIII

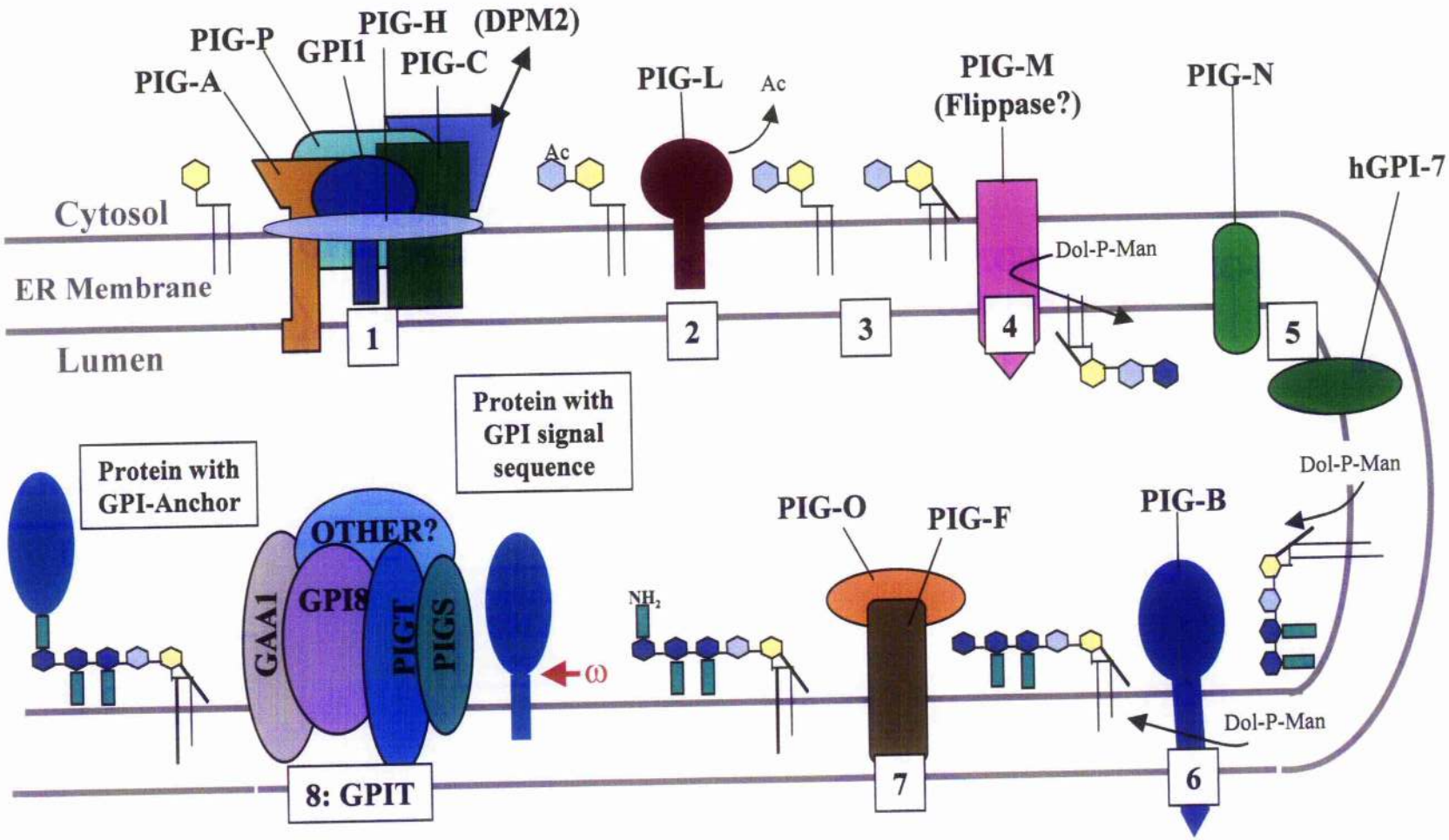
Two mannose residues are transferred from dol-p-man to the backbone in two independent reactions.

Step7: EtN-P Transferase

Addition of EtN-P to the final mannose

Step8: GPIT

The complete anchor is attached at the ω site of the awaiting GPI anchored protein, by the EtN-P residue on the GPI anchor, catalysed by the GPIT complex


PIG-P
PIG-H
(DPM2)
GPI1
PIG-C
PIG-A
PIG-L
Ac
PIG-M
(Flippase?)
PIG-N
hGPI-7
Ac
Cytosol
ER Membrane
Lumen
Dol-P-Man
1
2
3
4
5
Protein with
GPI signal
sequence
Protein with
GPI-Anchor
Dol-P-Man
PIG-O
PIG-F
PIG-B
OTHER?
GAA1
GPI8
PIGT
PIGS
NH2
ω
Dol-P-Man
8: GPIT
7
6

STEP	Mammalian	Yeast	Trypanosomes	
			T. brucei	*Leishmania*
1	PIG-A	GPI3		
1	PIG-C	GPI2		
1	PIG-H	GPI15		
1	hGPI1	GPI1		
1	PIG-P			
1	DPM2	DPM2		
2	PIG-L	GPI12	TbGPI12	GPI12
3			(GPIdeAc)	
4	PIG-M	PIG-M	PIG-M	
5a	PIG-N	MCD4	N/A	N/A
5b	hGPI-7	GPI7	N/A	N/A
6	PIG-B	GPI10	TbGPI10	
7	PIG-F	GPI11		
7	PIG-O	GPI13	(PIG-O)	
8	hGPI-8	GPI8	GPI8	GPI8
8	hGAA1	GAA1		
8	PIG-S	GPI16		
8	PIG-T	GPI17		

Table 1.1: Summary of proteins involved in GPI biosynthesis

Summary of proteins so far identified involved in GPI biosynthesis. The protein homologues found in mammalian, yeast and trypanosomes cells are listed. The step in the pathway with which each protein is associated is numbered according to the system used in the legend of figure 3.3, and can be directly related to the text in section 1.4.

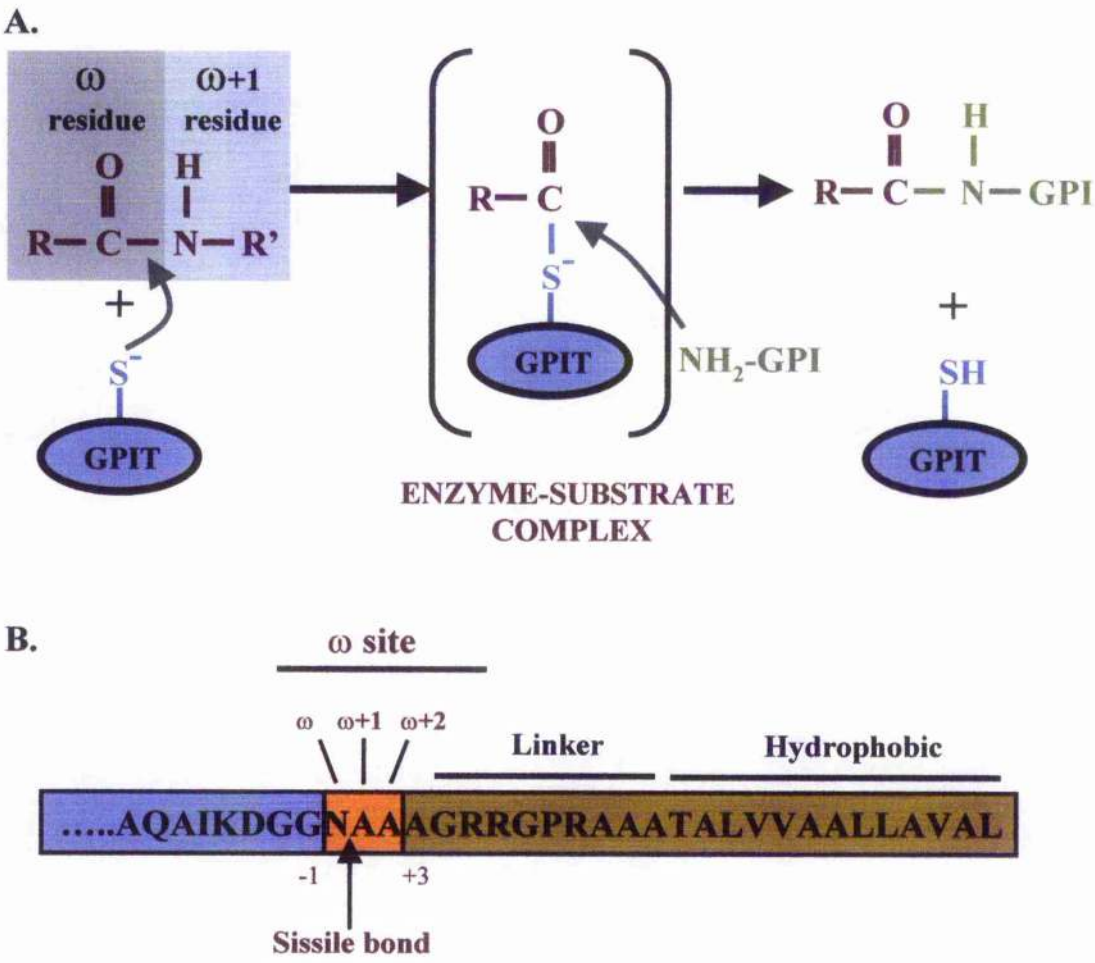


Figure 1.4: GPI anchor addition

A). The mechanism of GPI anchor addition. The carbonyl group at the ω site on the awaiting protein, is activated by a sulfhydryl group on the transamidase. An enzyme–substrate complex forms. The C-terminal end is cleaved from the protein. The complex undergoes nucleophilic attack by the amino group of the EtN-P residue present on the GPI anchor. The protein is linked to the GPI anchor by an amide bond, the GPIT enzyme has its active sulfhydryl residue restored (after Sharma *et al.*, 1999).

B). Summary of the structure of the C-terminal end of a GPI anchored protein. Hydrophobic, and linker regions are indicated as are the residues surrounding the point of GPI anchor addition (ω site). The peptide sequence is that of *L. mexicana* GP63.

Chapter 2

Materials and Methods

2.1 Bacteriology methods

2.1.1 Bacterial strains

XL1-Blue MRF', Stratagene.

2.1.2 Bacterial culture and long term storage

Cells were plated on LB-agar plates with appropriate antibiotics and incubated at 37°C overnight. A single colony was picked using a sterile toothpick and used to inoculate 10ml of LB broth containing appropriate antibiotics. The culture was grown overnight at 37°C in a rotary incubator (220rpm). The overnight culture was then either used directly for small-scale plasmid preps, or 1ml used to inoculate larger cultures. For long term storage 0.5ml of the overnight culture was mixed with an equal volume of 2% peptone/ 40% glycerol. Cells were stored at -80°C.

2.1.3 Preparation of heat shock competent cells

A 5ml overnight culture of cells was diluted 1/100 in 50ml of LB-broth and incubated at 37°C in a rotary incubator until an OD_{600} of 0.6 was reached. The culture was incubated on ice for 10 minutes, and harvested by centrifugation at 2000rpm for 15 minutes at 4°C. Cells were resuspended in 16ml of cold RF1, incubated on ice for 15 minutes, and pelleted by centrifugation at 1800 rpm, 4°C for 15 minutes. Cells were resuspended in 4 mls cold RF2, incubated on ice for 1hour, divided in to 200µl aliquots snap-frozen on dry-ice/ ethanol and stored at -80°C,

2.2 *Leishmania mexicana* methods

2.2.1 Leishmania mexicana cell lines and culture methods

The wild type *L. mexicana* cell line used throughout this study was *Leishmania mexicana mexicana* (MNYC/BZ/M379). Δ*gpi8* was derived from this cell line by Dr. Jim Hilley (Hilley *et al.*, 2000).

L. mexicana wild-type and Δ*gpi8* promastigotes were maintained in culture at 25°C in modified Eagle's medium (HOMEM) containing 10% (v/v) heat inactivated foetal calf serum (FCS). Neomycin (G418, Life technologies) was added at 25 μg ml^{-1} typically, and up to 500 μg ml^{-1} as required. Cells were inoculated into fresh medium at approximately 1 x 10^6 cells ml^{-1} and were passaged into fresh medium when cultures reached late-log or early stationary phase.

Cells were harvested by centrifugation at 2000xg for 5 minutes with 2 subsequent washes in PBS each followed by centrifugation as above. Cell pellets were stored at -80°C until required.

HOMEM plates were made by a 1:1 dilution of 2X HOMEM with 2% agar containing appropriate antibiotics. Plates were allowed to air dry in an airflow hood for 15 minutes and were pre-warmed to 25°C for 30 minutes before cells were spread over the solid medium. Liquid was allowed to absorb into the plate for 5 minutes before the plates were sealed with parafilm and incubated at 25°C.

2.2.2 Stabilate preparation and long term storage

0.5mls of log phase culture was diluted with an equal volume of fresh medium containing 10% DMSO in a Cryotube vial (Nunc). Samples were stored at -70°C overnight and transferred for long term storage under liquid nitrogen.

2.2.3 Transfection of *L. mexicana*

Transfection of *Leishmania* was carried out following the methods of Coburn and co-workers (Coburn *et al.*, 1991). *Leishmania* promastigotes were grown to a density of 0.9-1 x 10^7 cells ml^{-1}, harvested and washed twice in electroporation buffer (EPB), before resuspension at 1 x 10^8 cells ml^{-1} in EPB and kept on ice. 4 x 10^7 cells (400μl) were transferred to a pre-chilled 0.2cm electroporation cuvette (Biorad). 15μg of chilled, sterile DNA was added to the cuvettes as appropriate. Cells were electroporated using the Genepulser II apparatus with set to 0.45kV (2.25kV/cm), and 500μF capacitance, and incubated on ice for up to 10 minutes. Cells were transferred to fresh medium and incubated at 25°C overnight. Cells were plated out onto plates containing

appropriate antibiotics. Alternatively 1ml of transfected culture was transferred to a fresh flask and 9ml of medium containing the appropriate antibiotic added.

2.3 Tissue culture

2.3.1 Hybridoma cells

The Hybridoma cell line expressing the BB2 antibody (Brookman *et al.*, 1995), was grown in Serum Free Protein Free Hybridoma Medium (Sigma). When cells reached a density of 10^6 cells ml^{-1} they were passaged by diluting 1:10 in fresh culture medium. Spent medium was recovered for use as antibody in the detection of the TY epitope, by removing cells by centrifuging and filtering the medium. Recovered supernatant was stored at 4°C with 0.02% NaN_3.

2.4 Molecular methods

2.4.1 Plasmid purification

Plasmids were routinely purified from bacterial culture using Qiagen kits, following the manufacturers instructions. For small scale purification for use in subcloning or restriction digests Qiagen miniprep kits were used, for larger scale purification, such as for the transfection of *L. mexicana*, Qiagen Tip-20 kits were used. Plasmid quality and yields were assessed by spectrophotometry. An absorbence reading of 1 at a wavelength of 260nm is equivalent to 50μg ml^{-1} double stranded DNA.

2.4.2 Ethanol precipitation

Where required DNA was purified or concentrated by ethanol precipitation in 2.5 volumes 100% ethanol, 300mM NaAcetate (pH 5.2), on ice for 1 hour, and centrifugation at 15,000 rpm for 15 minutes. The supernatant was removed and the pellet washed twice in 70% ethanol, and then air dried for 15 minutes. DNA was re-suspended in an appropriate volume of H_2O.

2.4.3 Polymerase Chain Reaction (PCR)

PCR was used for the amplification of specific DNA fragments either for subsequent cloning or analysis, and the identification of positive transformants by whole-cell PCR. A 20μl reaction contained 2μl of 10x PCR buffer, 50ng of DNA template, 20pM of each primer, and 2U of Taq polymerase. Taq polymerase lacks proof reading ability

therefore Pfu polymerase was used where high fidelity PCR was required. Reaction conditions varied and are described where appropriate.

Taq amplified PCR products were ligated directly into the pGEM-T vector (Promega). Pfu generated fragments were cloned into PCR-script (Stratagene). Alternatively to clone Pfu generated products into pGEM-T, completed PCR reactions were heated to 96°C for 30 minutes, and then incubated at 72°C with 1U Taq polymerase, 200μM dATP for 2 minutes. This incorporated an additional adenosine overhang to the PCR products.

Colony PCR was used to screen for bacterial colonies containing correctly orientated inserts. A toothpick was touched to a specific colony, and then used to transfer cells directly to a 20μl PCR reaction lacking template DNA.

2.4.4 Restriction digests

Restriction enzymes were from New England Biolabs (NEB), and digests were set up following the manufacturers recommendations, and using the specific buffer supplied with each enzyme. Generally reactions were carried out for 1 hour at 37°C.

2.4.5 DNA gel electrophoresis

DNA was run out on gels containing 0.7- 1.2% agarose in TAE buffer. Samples were mixed with 5 x DNA gel loading buffer to give a final concentration of 1x loading buffer and electrophoresed at 50- 110V, until the dye in the loading buffer had migrated two thirds of the length of the gel. 1kb ladder (Gibco) was used as a marker and generally 0.5μg was loaded per lane. Ethidium bromide was added to molten gel at a final concentration of 0.3μg ml^{-1}, unless uniform staining was required in which case after electrophoresis the gel was soaked in TAE buffer containing 0.3μg ml^{-1} ethidium bromide for 30 minutes, and washed in TAE buffer. Gels were visualised with an UV light using the Gel Doc 2000 imaging system (BioRad), and analysed using Quantity One (Biorad).

2.4.6 Purification of DNA from agarose gels

DNA fragments were purified from TAE agarose gels, using a sephaglass gel purification kit (Amersham) following the manufacturers instructions. DNA was finally eluted from the sephaglass in 10µl of sterile water.

2.4.7 DNA ligation

Ligation of PCR fragments into commercially available vectors such as pGEM-T or PCR-Script was carried out following the manufacturers instructions. For ligation of DNA fragments into other vectors, the plasmid and insert were mixed at a ratio of approximately 1:3 in a 10µl reaction containing 1x T4 DNA ligase buffer, and 200U of T4 DNA Ligase (NEB). Reactions were incubated at 16°C overnight.

2.4.8 Transformation of competent bacteria

Competent cells were heat shocked as follows: An aliquot of competent cells was thawed on ice, and 40µl of cells was aliquoted into fresh tubes for each transformation. 1µl of ligation mix was added to the cells and incubated on ice for 1 hour. Cells were heated to 42°C for 45 seconds and returned to ice for a further 2 minutes, and then transferred to a tube containing 1ml of pre-warmed SOC medium and allowed to recover for 1 hour at 37°C in a rotary incubator. 50-200µl of transformed cells were plated out onto LB-agar plates containing appropriate antibiotics, and incubated at 37°C overnight.

2.4.9 DNA sequencing

DNA sequencing was carried out by the University of Glasgow Molecular Biology Support Unit (MBSU). 500ng of DNA template, and 3.2pM of the appropriate primer were supplied.

2.5 Biochemical methods

2.5.1 SDS-PAGE

Whole cell lysates of promastigotes were prepared by the harvesting of 10^8 cells. The cell pellet was lysed in 75µl of 0.25% Triton X-100 on ice, and 25µl of 4X SDS-PAGE loading buffer was added and the samples boiled for 5 minutes.

Proteins were separated by SDS-PAGE, typically on 12% acrylamide gels, in Tris-Glycine buffer. Pre-stained molecular weight protein standards were used as size markers, either MultiMark (Novex), or SEE-blue markers (Invitrogen). Mini gels of 0.75mm thickness were electrophoresed using the Mini-Protean system (BioRad), and larger gels of 0.8mm thickness were run using Gibco vertical gel apparatus. To visualise total protein gels were stained with Coomassie blue, and washed with destain, alternatively gels could be electroblotted. Gels containing radioactive samples were Coomassie stained, and then fixed in destain for 30 mins before drying on a vacuum dryer (BioRad) 80°C for 2 hours.

2.5.2 Western blotting

Following SDS-PAGE proteins were transferred to PVDF membrane by electroblotting using a BioRad semi dry blotter following the manufacturers instructions. The PVDF membrane was pre-soaked for 5 minutes in methanol, and the gel rinsed briefly in Western Blot Transfer Buffer prior to transfer. Typically for a single Mini-Gel, transfer conditions were 0.1 amps for 30 minutes. After transfer the membrane was transferred to Blocking solution and incubated either at 4°C overnight, or at room temperature for 1 hour both on a rolling platform. In some cases membranes were rinsed in Ponceau S (Sigma) to visualise protein transfer. Membranes were washed for 20 minutes in PBS-Tween prior to antibody detection.

2.5.3 Gelatin activity gels

Gelatin gels were prepared as for normal 10% SDS-PAGE with the addition of 0.1% gelatin (v/v) to the resolving gel, added from a 2% gelatin stock preheated prior to gel preparation. Protein samples were prepared for loading on gelatin gels by addition of 4x SDS-PAGE loading buffer to a final concentration of 1x. Samples were not boiled prior to loading on the gel. Gelatin gels were electrophoresed using the buffer and conditions described for SDS-PAGE. Gels were subsequently treated by soaking in 2.5% TX-100 for 30 minutes to remove excess SDS, and rinsed in MilliQ water. Activity was assessed by incubation at 37°C overnight in an appropriate buffer, as described in the text. Activity was visualised by staining with Coomassie and destaining.

2.5.4 Antibody detection of proteins

Primary antibodies were incubated with the membranes in appropriate blocking solution for 1 hour at room temperature. The membrane was washed 3x 10 minutes in PBS-Tween, and incubated with secondary antibody in Blocking solution for 30 minutes at room temperature. Blots were washed 3 x 10 minutes in PBS-Tween. The dilution of the primary antibody was as appropriate to the specific antibody used. The secondary antibody was either anti-mouse IgG or anti-rabbit IgG both conjugated to horseradish peroxidase (HRP) (Promega), and was typically used at a dilution of 1 in 5000. Antibody binding was detected by Supersignal Enhanced Chemiluminescence (ECL) detection kit (Pierce) following the manufacturers instructions. The signal was detected using autoradiography film (NEN), and the film developed by Kodak X-omat automated developer.

2.5.5 Immunofluorescence microscopy

30µl of late log *L. mexicana* promastigote cell culture were taken and diluted to 100µl in PBS. 20µl were dropped on to slides, smeared over and allowed to air dry. The cells were fixed with in 2.5% paraformaldehyde, PBS for 15 minutes, and washed in PBS before the addition of primary antibody diluted as appropriate in PBS. The slides were incubated in a humidifying chamber for 30 minutes, washed in several changes of PBS, blocked in 10% FCS/ PBS for 30 minutes, then washed as before in PBS. A mixture of secondary antibody diluted as appropriate with PBS, and DAPI (4,6-diamidino-2-phenylindole) 20µg ml^{-1}, was applied, and the slides incubated in a humidifying chamber in the dark for 30 minutes. Slides were washed in PBS, and 25µl of anti-quenching agent MOWIOL-DABCO was added underneath a glass cover slip. Cells were viewed by UV fluorescence microscopy on a Zeiss Axioplan fluorescence microscope. Images were captured using a Hamamatsu digital camera, and OpenLab software (Improvision, University of Warwick).

2.5.6 Fluorescence microscopy of live cells

L. mexicana promastigotes expressing GFP were grown to mid log phase, and 1 ml of culture taken and the cells washed twice in PBS. The cell pellets were resuspended in 0.5 ml PBS and NaN_3 added to a final concentration of 0.005mM. Cells were

transferred onto a slide and analysed for GFP expression by UV fluorescence microscopy using a Zeiss microscope as previously described.

2.5.7 Metabolic labelling

L. mexicana promastigotes were grown to mid log phase, washed twice in PBS and 6 x 10^7 cells resuspended in 1ml of labelling medium (1x Minimum Essential Medium (ICN), 2mM L-glutamine, 10% (v/v) dialysed FCS), and 100µCi of ^{35}S-ExpreSS ([^{35}S] Methionine/ cysteine Protein Labelling Mix (NEN)). Cells were grown at 25°C for 6 hours, washed 3 times in ice cold PBS and the cell pellets and medium fractions were stored at -80 °C prior to analysis. For pulse chase labelling experiments, 3.6 x 10^7 cells were resuspended in 100µl labelling medium containing 100µCi of ^{35}S-ExpreSS per time point. Cells were labelled for 12 minutes at 25°C, washed 3 times in ice cold PBS and resuspended in an equivalent volume of HOMEM containing 10% (v/v) FCS at 25°C. 100µl aliquots of cells were removed at appropriate time points, and the cells and medium stored separately at -80 °C in the presence of protease inhibitors (1mM EDTA, 200µg ml^{-1}, Pefabloc SC, 5 µg ml^{-1} pepstatin A, 40 µg ml^{-1} leupeptin, 200µM PMSF, 1mM phenanthroline)

2.5.8 TX-114 fractionation

This was carried out following the method of Bordier (Bordier, 1981). Briefly cells were lysed in 200µl TX-114 buffer on ice for 15 minutes, precleared to remove cellular debris by centrifugation at 10,000g for 10 mins at 4 °C, and the supernatant overlaid onto a sucrose cushion (10mM Tris-HCl pH7.4, 150mM NaCl, 6% w/v sucrose, 0.06% pre-condensed TX-114) in a fresh eppendorf tube. Samples were incubated at 30°C for 3 minutes, centrifuged at 300g for 3 mins at RT, and the upper aqueous layer removed to a fresh tube. 0.5% pre-condensed TX-114 was added to this sample, incubated at 4°C for 10 minutes, and then this upper layer was overlayed back on the original sucrose cushion. The sample was incubated at 30°C for 3 min, centrifuged for 3 minutes at 300g at room temperature. The whole aqueous phase was removed to a fresh tube, leaving a small TX-114 pellet containing the membrane fraction. The aqueous phase was treated with 2% pre-condensed TX-114, incubated at 4°C for 10 minutes, transferred to 30°C for three minutes and centrifuged at 300g at RT for 3 minutes. The

aqueous phase containing the soluble cell fraction was then transferred to a fresh tube, and the soluble and membrane fractions subjected to further analysis.

2.5.9 $NaCO_3$ Extraction

Pellets containing 3 x 10^7 *L. mexicana* promastigotes were resuspended in 50µl Lysis Buffer 1 (H_2O containing protease inhibitors), 50µl of Lysis Buffer 2 (100mM HEPES, 50mM KCl, 10mM $MgCl_2$, 20% (v/v) glycerol and protease inhibitors) snap frozen, thawed and vortexed to lyse the cells. Cells were centrifuged at 15,000 rpm for 15 minutes to remove cell debris, the supernatant transferred to a fresh tube, 450µl of 100mM $NaCO_3$ (pH 11), and protease inhibitors added, vortexed and incubated on ice for 30 minutes. The 500µl samples were transferred to ultracentrifuge tubes, and 100µl of sucrose cushion (0.5M sucrose, 100mM $NaCO_3$ (pH 11), and protease inhibitors) was pipetted carefully to the bottom of the tube. Samples were centrifuged at 83,000g for 30 minutes. The upper layer was removed as the aqueous fraction, while the pellet was removed as the membrane fraction, and treated by immune-precipitation.

2.5.10 Immune-precipitation

This was carried out with GPI8 antibody R492 (Hilley, 1999) following the method of Sharma and co-workers (Sharma *et al.*, 2000). Briefly, cell pellets were resuspended in 1 x GPI8 solubilization buffer and protease inhibitors, to 1 ml, and centrifuged at 14,000 rpm for 15 minutes to pre-clear. 50 µl of protein A/G sepharose (resuspended to a concentration of 0.5mg ml^{-1} in solubilization buffer) with 6µl αGPI8, were added to the supernatant and the samples mixed at 4°C for 12 hours. In some cases αGPI8 was replaced with 6µl of GPI8 pre-immune serum. Samples were then washed 3 times in TEN-D and once in TEN buffer (TEN-D buffer in the absence of detergent). 25µl of 2 x SDS loading buffer was added, the samples boiled prior to analysis by SDS-PAGE. For immune-precipitation of Green fluorescent protein (GFP) chimeras, the same method was used, but 4µl of Living Colors GFP monoclonal antibody, JL-8, (Clontech), was used in place of the GPI8 antibody.

For immune-precipitation of GP63 antibody L3.8 was used (Medina-Acosta *et al.*, 1989) Cells were lysed in 1ml IDB and protease inhibitors, and precleared. Medium samples had an equivalent volume of 2 x IDB buffer added, and were then made up to

1ml with 1 x IDB buffer. 100µl of Protein G Sepharose (resuspended to 0.5 mg ml^{-1} in IDB buffer) and 10µl L3.8 antibody were added to the samples, incubated for 12 hours at 4°C, and the beads subsequently washed three times in GP63 wash buffer, and once in TEN. 40µl of 2x SDS-Page loading buffer was added to the samples prior to further analysis.

2.5.11 PI-PLC digestion

Cells were pulse chase labelled, and 2 samples collected for each time point, washed 3 times in ice cold PBS and snap frozen. These samples were lysed in 200µl TX-114 buffer but with the absence of TX-114 and the addition of 0.05% TX-100, incubated at room temperature for 10 minutes, and centrifuged to pre-clear. Samples were transferred to a fresh tube, and 4µl of PI-PLC (Sigma) added to one sample from each time point. All samples were incubated at 37°C for 1 h. 0.5% pre-condensed TX-114 was added to each sample, and the samples incubated at 4°C subsequent to TX-114 fractionation as previously described.

2.5.12 PNGaseF digestion

Samples to be PNGaseF treated were TX-114 fractionated, and GP63 immune-precipitated as previously described. GP63 samples associated with protein G beads were spilt into 2 and one half treated with 2 µl PNGaseF (NEB), and the other mock treated following the manufacturers instructions.

2.5.13 Purification of GP63 on Concanavlin-A

Cells were grown to stationary phase, the cells harvested, and the medium filtered and retained. Cells were lysed in 1 x Con-A binding buffer for 30 mins, centrifuged to preclear, and 500µl of ConA sepharose (resuspended to 4 mg ml^{-1} in ConA binding buffer), and protease inhibitors (200 µg ml^{-1} Pefabloc SC, 5 µg ml^{-1} pepstatin A, 40 µg ml^{-1} leupeptin) added. To the medium 1 vol of 2 x ConA binding buffer, 500µl of ConA sepharose and protease inhibitors were added. Samples were mixed for 12 h at 4°C, washed 4 times in ConA binding buffer, and glycosylated proteins eluted with an appropriate volume of elution buffer (1M methyl α-D-mannopyranoside in ConA binding buffer).

2.5.14 Protein G purification of antibody

1.5 mls of packed Protein G beads (Sigma) were washed 2x in binding buffer (20mM sodium phosphate , 150mM NaCl (pH 7.4)) and resuspended to a final volume of 10 mls in binding buffer and packed onto a 10ml column (Pierce). The column was washed with 10 column volumes (cv) of binding buffer. 50 mls of BB2 hybridoma cell supernatant was allowed to drip slowly through the column, and washed with 10 cv of binding buffer. Antibody was eluted with 6 mls of elution buffer (100mm Glycine-HCl pH3.0), in 500µl fractions and each fraction neutralised with 50µl 1M Tris-HCl pH 9.0. Fractions were assessed by spectrophotometry for protein content. An absorbance reading of 1 at a wavelength of 280nm is equivalent to 800µg ml^{-1} of protein.

2.6 Buffers and reagents

Antibiotics: Ampicillin- Stock: 100mg ml^{-1} in distilled water, used at 100µg ml^{-1}.

Kanamycin- Stock: 25mg ml^{-1} in distilled water, used at 25µg ml^{-1}.

Acrylamide gel:

Resolving Gel

	Acrylamide gel		Gelatin
	12%	10%	10% acrylamide/ 0.1% gelatin.
dH_2O	3.35 ml	4.00 ml	3.5 ml
1.5 M Tris-HCl, pH8.8	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl	100 µl
Acrylamide/ Bis (30%)	4.0 ml	3.3 ml	3.3 ml
2% (w/v) gelatin	-	-	0.5 ml
10 % ammonium persulfate	50 µl	50 µl	50 µl
TEMED	10 µl	10 µl	10 µl

Stacking gel

dH_2O	6.1 ml
0.5M Tris-HCl, pH6.8	2.5 ml
10% (w/v) SDS	100 µl
Acrylamide/ Bis (30%)	1.3 ml
10% ammonium persulfate	75 µl
TEMED	10 µl

Blocking solution: 5% w/v Marvel, 0.01% v/v Tween, in PBS.

Brefeldin A: Stock is 10mg ml^{-1} in methanol.

Con-A binding buffer: 10mM Tris-HCl pH 7.4, 0.5M NaCl, 0.5% TX-100, 1mM $CaCl_2$, 1mM $MnCl_2$.

Coomassie: 2.5g Coomassie Brilliant Blue R-250 dissolved in 400ml methanol, 500ml H_2O, 100ml acetic acid.

DNA gel loading buffer: 0.25% (w/v) bromophenol blue, 30% v/v glycerol in H_2O.

Destain: 40% methanol, 10% acetic acid.

Electroporation Buffer (EPB): 21mM HEPES pH7.5, 137mM NaCl, 5mM KCl, 0.7mM phosphate buffer, 5mM glucose. Store at 4°C.

GPI8 Solubilization Buffer: 50mM Tris-HCl, pH7.5,150mM NaCl, 5mM EDTA, 1%NP40, and protease inhibitors.

GP63 wash buffer: 0.1% TX-100, 0.02% SDS, 150mM Tris HCl pH 7.5.

IDB buffer: 1.25% TX-100, 190mM NaCl, 60mM Tris HCL pH7.5, 6mM EDTA and protease inhibitors.

Luria-Bertani (LB) agar: LB broth plus 0.8% (w/v) agar.

LB-broth: 1% bactotryptone (Difco), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl. Made up in distilled water and autoclaved for sterilisation.

MOWIOL-DABCO: 6g glycerol, 2.4g MOWIOL 4-88 (Calbiochem) in 6ml distilled water. 12ml 0.2M Tris-HCl, pH8.5 was added and incubated with agitation for several hours, incubated at 50°C for 10 minutes, then centrifuged (5000 g, 15 minutes). 1,4-diazobicyclo[2.2.2.]octate (DABCO) (Sigma), was added to 0.1% (w/v) final concentration and divided into 1ml aliquots, and stored at -20°C.

PBS: 10mM phosphate buffer, 2.7mM KCl, 137mM NaCl, pH 7.4.

PBS-Tween: 0.01% Tween v/v, PBS.

PCR-Mix (10x): 450mM Tris-HCl pH 8.8, 110mM $(NH_4)_2SO_4$, 45mM $MgCl_2$, 67mM β-mercaptoethanol, 44mM EDTA (pH 8.0), 10mMdCTP, 10mM dATP, 10mM dGTP 10mM dTTP, 113μg ml^{-1} BSA. Stored at -20°C.

Protease inhibitors: Generally 1mM EDTA, 200μg ml^{-1} Pefabloc SC, 5 μg ml^{-1} Pepstatin A, 40 μg ml^{-1} leupeptin, 200μM PMSF, 1mM phenanthroline unless otherwise indicated.

RF1: 100mM RbCl, 50mM $MnCl_2.4H_2O$, 30mM K Acetate, 10 mM $CaCl_2$, 15% glycerol, pH 5.8.

RF2: 10 mM MOPS, 10mM RbCl, 75mM $CaCl_2.4H_2O$, 15% glycerol, pH6.8.

SDS-PAGE loading buffer (4x): 200mM Tris-HCl pH6.8, 400mM β-mercaptoethanol, 8% SDS, 40% glycerol, 0.2% (w/v) bromophenol blue.

TAE (1 x): 40mM Tris acetate, 1mM EDTA.

TBS: 20mM Tris, 137 mM NaCl pH7.6.

TEN-D: 50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 1% NP40, 0.1% SDS, 0.5% deoxycholate.

Tris Glycine buffer (1x): 25mM Tris, 250mM glycine (pH 8.3), 0.1% SDS.

Tunicamycin: Stock is 2mg ml^{-1} in 25mM NaOH.

TX-114: TX-114 was prepared by pre-condensation after the method of Bordier (Bordier *et al.*, 1981). 1ml of TX-114 (Sigma) was made up to 50mls in 10mM Tris-HCl pH 7.4, 150mM NaCl, incubated at 0°C for 12 hours, and transferred to 30°C for 12 hours. The upper aqueous phase was removed and replaced with the same volume of 10mM Tris-HCl pH 7.4, 150mM NaCl, and the process repeated x 2. The final lower phase was stored at 4°C as the TX-114 stock solution, and was at a concentration of 11.4% (w/v).

TX-114 buffer: 10mM Tris-HCl pH 7.4, 150mM NaCl, 0.5% pre-condensed TX-114.

Western blotting transfer buffer: 5mM Tris, 2mM glycine, 20% methanol.

Chapter 3

Characterisation of *Leishmania mexicana GPI8*

3.1 Introduction

The addition of a complete GPI anchor close to the C terminal end of a GPI-anchored protein occurs in the ER lumen by the simultaneous cleavage of the protein at the GPI anchor attachment site and addition of the pre-formed GPI anchor. The GPI anchor is attached to the protein by amide linkage between the anchors terminal EtN-P group, and the C-terminal carboxyl group of the protein (Ferguson *et al.*, 1988). This reaction is catalysed by an enzyme with transamidase activity.

The GPI: Protein transamidase (GPIT) is a complex which has been well characterised in yeast and human cells, and contains at least 4 components. In yeast these complex members comprise GAA1 (Hamburger *et al.*, 1995), GPI16, (Fraering *et al.*, 2001) GPI17 (Ohishi *et al.*, 2001), and GPI8, which has a single membrane-spanning domain, and is located within the lumen of the ER (Benghezal *et al.*, 1996). GPI8 is considered to be the catalytic sub-unit of the GPIT complex, and directly cleaves the GPI attachment signal peptide.

The GPIT complex of trypanasomatids has been less well characterised. The *L. mexicana* GPI8 has been cloned, it has no apparent transmembrane domain, as predicted by sequence homology and hydrophobicity, and is considered a soluble homologue of yeast and mammalian GPI8 (Hilley *et al.*, 2000). An alternative interpretation of the protein sequence is provided by use of the TOPPRED2 programme (Eisenhaber *et al.*, 2001). This predicts that a C-terminal transmembrane domain exists between residues 251 and 271 of *L. mexicana* GPI8. The authors propose that the strict structural conservation found amongst the GPI8's from yeast and humans is unlikely to be deviated from in the case of *L. mexicana*, and the apparent absence of a transmembrane domain requires further investiagation (Eisenhaber *et al.*, 2001). The *T. brucei* GPI8 has also recently been cloned (Kang *et al.*, 2002; Lillico *et al.*, 2003). No transmembrane domain is predicted at the C-terminus of this protein (Kang *et al.*, 2002). *T. brucei* GPI8 has been demonstrated to be soluble as it can be removed from

trypanosome membranes by a high pH wash (Sharma *et al.*, 2000). No homologue of other transamidase complex members has yet been cloned from trypanasomatids, though analysis of sequence data has identified a possible GAA1 homologue in *L. major* (Eisenhaber *et al.*, 2001).

3.1.1 Previous work on *L. mexicana* GPI8

The work in this thesis continues directly on from a previous study (Hilley, 1999), and the results from this work are briefly summarised. The catalytic subunit of the GPIT of *Leishmania mexicana*, GPI8, was cloned and partially characterised (Hilley, 1999; Hilley *et al.*, 2000). The predicted protein was shown to share 31% homology with yeast and human homologues (Hilley *et al.*, 2000), and also shares significant homology with *T. brucei* GPI8 (Kang *et al.*, 2002; Lillico *et al.*, 2003). *GPI8* was demonstrated to be a single copy gene in *L. mexicana*, and a *GPI8* null mutant (Δ*gpi8*) was produced by targeted gene replacement. The Δ*gpi8* cell line was viable in culture, and was demonstrated by immunofluorescence analysis to lack GP63 from the cell surface. Introduction of an episomal copy of GPI8 (pGL269) into the Δ*gpi8* cells (generating the cell line Δ*gpi8*[pX*GPI8*]) restored GP63 to the cell surface. GPI anchor precursors accumulated in the mutant cells (Hilley *et al.*, 2000). *In vitro* studies demonstrated that Δ*gpi8* were able to infect, and replicate within, macrophages at a level similar to wild type cells. *In vivo* studies demonstrated that the mutants were also able to infect mice and cause lesions. It was concluded that GPI-anchored proteins of *L. mexicana* promastigotes were not essential for growth in culture, the invasion of macrophage, or the infection of mice. Nor were the GPI-anchored proteins required for the differentiation of promastigotes to amastigotes in macrophage or mice, and the subsequent survival of the amastigotes.

Polyclonal antibodies (R491/ R492) were raised against a recombinant GPI8 (A His-tagged form expressed in *E. coli*), and a second set of antibodies was raised against a GPI8 peptide (R771/ R455). Whilst both sets of antibodies were able to detect recombinant GPI8 in *E. coli* cell lysates, neither pair was able to detect GPI8 in lysates from *L. mexicana* cells, probably due to the low abundance of GPI8 in these cells. However, the GPI8His antibodies were able to detect a protein of the expected size in *T. brucei* cell lysates. Immunofluorescence analysis with the R491 GPI8His antibody

on *T. brucei* cells identified a protein which co-localised with the ER protein BiP, (Hilley, 1999), and the ribosomal protein QM (Lillico *et al.*, 2002).

An episome expressing a GPI8-GFP fusion protein was also produced (pGL190). The construct was engineered such that GFP replaces the stop codon at the 3' end of *GPI8*, and therefore is located at the C-terminus of GPI8. Western blot analysis suggested that the GPI8-GFP fusion protein was able to partially rescue the Δ*gpi8* mutant cell line (Hilley, 1999).

The work described in this thesis continued the characterisation of *L. mexicana* GPI8, and utilised the tools produced by the previous study. The aim of this section of work was to identify the GPI8 active site residues, and analyse the mutants produced, in order to further characterise *L. mexicana* GPI8.

3.2 Identification and analysis of *L. mexicana* GPI8 active sites

Previous studies provided some indication of the possible candidate residues which may act as the active site of *L. mexicana* GPI8. A cell-free assay for GPI anchoring in trypanosomes has been used to establish a reaction mechanism for GPI anchor addition (Sharma *et al.*, 1999b) described in section 1.4.8. The small nucleophile hydrazine can substitute for the GPI moiety within the transamidase reaction, and cause the release of VSG into the medium. Sulfhydryl alkylating reagents inhibit this reaction. Transamidase activity can be reconstituted in *T. brucei* membranes depleted of transamidase activity by addition of recombinant *L. mexicana* GPI8. This activity is inhibited when *L. mexicana* GPI8 is first incubated with sulfhydryl alkylating agents (Sharma *et al.*, 2000). Together, these data indicate that GPI8 contains a catalytically important cysteine residue.

Sequence analysis of yeast, human and *Leishmania* GPI8 showed that the proteins share significant homology to a unique family (C13) of cysteine proteases known as legumains (Benghezal *et al.*, 1996; Hilley *et al.*, 2000; Ohishi *et al.*, 2000). These proteases are characterised by a catalytic dyad comprising a histidine and a cysteine residue (Barrett and Rawlings, 1996). The C13 and GPI8 families now exist as two subfamilies (Meyer *et al.*, 2000).

This evidence suggests that a sulfhydryl group acts as the active site nucleophile for GPIT, and the active site of *L. mexicana* GPI8 is a cysteine residue. It also seems likely that the cysteine acts as a catalytic dyad in conjunction with a histidine, similar to other members of the C13/ GPI8 sub-families. Sequence homology has identified conserved cysteine and histidine residues in the GPI8 family (Hilley *et al.*, 2000).

3.2.1 Site specific mutagenesis of potential active site residues

Sequence alignment of *L. mexicana* GPI8 with other members of the GPI8 subfamily, Human, yeast and *T. brucei*, identified 4 conserved amino acids H63, C94, H174 and C216, which were potential active site residues (Figure 3.1). Each of these residues was individually mutated by site directed mutagenesis of the episomal copy of *L. mexicana* GPI8, pXGPI8 (pGL269), using the QuikChange Site Directed Mutagenesis Kit (Stratagene). pGL269 was previously prepared by cloning GPI8 and its 5' and 3' flanking regions into the pXG episomal expression vector (Ha *et al.*, 1996), and is 9.96kb in size (Hilley *et al.*, 2000).

The QuikChange Site Directed Mutagenesis Kit is a PCR based method that was used following the manufacturer's directions. The primer pairs for site directed mutagenesis (Table 3.1) were designed to mutate GPI8 at specific amino acids, with cysteine residues mutated to glycine, and the histidine residues converted to alanine (Hilley, 1999). Briefly, a 50µl PCR reaction was set up containing 10ng of plasmid DNA, 125ng of each of the appropriate primers, dNTPs, 1 x buffer and 2.5U Pfu Turbo, a DNA polymerase with proof reading capabilities. The elongation time for the PCR reaction was set at 20 minutes (2 minutes/ 1kb of plasmid template). Subsequent to the PCR, the products were treated with 10u of *Dpn*I, at 37°C for 1 hour. This was to remove parental plasmid DNA. 1µl of each PCR reaction was then transformed into 40µl of XL-1 blue competent cells, and plated out onto LB plates containing ampicillin. After overnight incubation at 37°C, approximately 250 colonies were present for each PCR reaction. Subsequent to plasmid purification, sequencing was used to confirm that the plasmids contained the correct mutations, and the plasmids were named pGL449 (H63A), pGL450 (C94G), and pGL451 (H174A). To confirm that only the intended mutation had been introduced by the PCR method into each plasmid, the entire *GPI8* ORF was checked by sequencing. Plasmid DNA was purified using a Qiagen Tip 100,

and 15μg of DNA was used to transfect the *L. mexicana* Δ*gpi8* cell line. The resulting cell lines were termed Δ*gpi8*[pX*gpi8*^{H63A}], Δ*gpi8*[pX*gpi8*^{C94G}] and Δ*gpi8*[pX*gpi8*^{H174A}]. The cell line Δ*gpi8*[pX*gpi8*^{C216G}] was generated in a previous study (Hilley, 1999).

Primer	Mutation	Sequence (5'→3')	Plasmid
OL330	**His^{63}→Ala^{63}**	CTCTTCAACTACCGC**GC**CACCGCCAATGCGC	pGL449
OL332	**Cys^{94}→Gly^{94}**	GACAGCTTCGCC**G**GCGACCCGCGAAATG	pGL450
OL334	**His^{174}→Ala^{174}**	CTACGTCGCGGGG**GC**CGGCGCCAAGTC	pGL451
OL336	**Cys^{216}→Gly^{216}**	CCTGGCAGATACA**G**GCCATGCGATTGCG	pGL403

Table 3.1: Primers used to mutate potential active site residues of GPI8
Mutated nucleotides are shown in bold and shaded grey. For each mutation a second complementary primary was also used. The plasmid pGL403 was produced by Hilley, (1999).

3.2.2 Episomal expression in *L. mexicana* Δ*gpi8* cell lines

To confirm that GPI8 was expressed from the episomes transfected into the *L. mexicana* cell lines, a GPI8 antibody (R492) was used. This antibody was demonstrated in a previous study to be unable to detect *L. mexicana* GPI8 by western blotting of WT, Δ*gpi8*, and Δ*gpi8*[pX*GPI8*] (Hilley, 1999). The antibody was therefore used to immune-precipitate proteins from metabolically labelled cells, as this was regarded as a more sensitive approach.

To confirm the R492 antibody was suitable for immune-precipitation of GPI8, the cell lines WT, Δ*gpi8*[pX*GPI8*], Δ*gpi8*[pX*gpi8*^{C216G}], and Δ*gpi8*[pX*GPI8*-GFP] were metabolically labelled with [^{35}S]ExpreSS protein labelling mix (NEN), for 6 hours in labelling medium prior to immune-precipitation. The cell lysates were immune-precipitated with either 6μl of pre-immune serum as a negative control, 6μl of αGPI8

or 5µl of αGFP (Clonetech), and protein A/G sepharose beads following the method of Sharma *et al.*, (2000) as described fully in Chapter 2. Samples were electrophoresed on a 12% SDS-PAGE gel, and the gel dried down and exposed to film, or visualised on a Typhoon phosphor imager.

Immune-precipitation of WT cells with a GPI8 antibody detected a protein of size 42kDa with both the pre-immune and immune serum (Figure 3.2 A, Lanes 1 and 2). Immune-precipitation from the cell line expressing $GPI8^{C216G}$ from an episome (Lanes 5 and 6) also detected a 42kDa protein using both pre-immune, and immune serum. However, the protein precipitated with the immune serum was greatly enriched when compared to the pre-immune immune-precipitation. This suggested the pre-immune serum detected a small amount of an unspecified 42kDa protein, whilst the immune serum detected a specific 42kDa protein. There was no enrichment of the 42kDa protein in WT cells when pre-immune and immune serum were compared, this suggested that a specific protein could not be detected in these cells.

A specific protein of size 42kDa was immune-precipitated from the GPI8 and $GPI8^{C216G}$ expressing cells (Lanes 6 and 8), whilst from the cell line expressing the GPI8-GFP fusion protein, a protein of size 72kDa was immune-precipitated with both the αGPI8 polyclonal antibody and the commercially available αGFP antibody (Lanes 4 and 10). Precipitation with the pre-immune serum produced no proteins of comparable intensity (Lanes 3, 5, 7 and 9) suggesting that immune-precipitation with the antibody was protein specific.

The predicted size of GPI8 is 42kDa, and the predicted size of the GPI8-GFP fusion protein is 75kDa. The R492 polyclonal GPI8 antibody, therefore, is able to specifically immune precipitate a protein of the predicted size of GPI8 or GPI8-GFP from cell lines expressing episomal copies of these proteins. The use of the GFP antibody demonstrates that a protein of the expected size (72kDa) was immune-precipitated from the GPI8-GFP expressing cell line, comparable in size to that precipitated using the GPI8 antibody. This further demonstrates that the protein that the R492 antibody is detecting is GPI8. This experiment confirmed the validity of using immune-precipitation with the R492 antibody, as a method for detecting GPI8.

The R492 polyclonal GPI8 antibody was then used to confirm GPI8 expression in all cell lines expressing different mutant forms of GPI8 from an episome (Figure 3.2 B). As expected, no GPI8 protein was detected in the Δ*gpi8* cell line (Lane 2). However, as in the previous experiment, no GPI8 was detected in WT cells (Figure 3.2 A, lane 2 and Figure 3.2 B lane 1). This is an indication of the low level of expression of GPI8 in wild type cells, and suggests that re-expression from an episome vastly over-expresses GPI8 compared to normal levels. A protein of size 42kDa, the predicted size of GPI8, was detected in all cell lines re-expressing the different forms of GPI8 (Figure 3.2 B, lanes 3-7).

3.2.3 GPI8 activity in active site mutants

The effect of the 4 mutations on *L. mexicana* GPI8 activity were assessed by examining the effect on GP63, the major GPI-anchored protein of *Leishmania* promastigotes. The Δ*gpi8* line itself has been shown to be deficient in the major GPI-anchored surface protein of *L. mexicana*, GP63 (Hilley *et al.*, 2000). Re-expression of *GPI8* in Δ*gpi8* (the Δ*gpi8*[pX*GPI8*] line) restored GPI-anchored GP63 to the cell surface. Western blot and immunofluorescence analysis with antibodies specific to GP63, were used to assess the effect of the *GPI8* mutations on the ability of the GPIT complex to add GPI-anchors onto GP63. GP63 could not be detected in cell lysates prepared from the GPI8^{C216G} expressing cell line, though expression of GPI8 in this cell line had not previously been confirmed (Hilley, 1999).

3.2.3.1 Western blot analysis of GP63 expression

Western blot analysis was used to study the effect of the GPI8 mutations on GP63 in total cell lysates (Figure 3.3). Cell lysates containing 10^8 cells grown to mid to late log stage, were prepared, and 10^7 cell equivalents were electrophoresed by 12% SDS PAGE. The gel was electroblotted prior to antibody detection of GP63. The primary antibody used was a mouse monoclonal, raised against *L. major* GP63, a gift from Dr. Robert McMaster, University of British Columbia, Vancouver, Canada (Button *et al.*, 1991). The antibody has been demonstrated to cross react with *L. mexicana* GP63 in western blotting, but is unable to detect GP63 in its native form in immunofluorescence experiments. The primary antibody was used at a dilution of 1:50; the secondary antibody, an anti-mouse IgG-horseradish-peroxidase conjugate (Promega), was used at

a dilution of 1:5000. Antibody detection was by ECL (Pierce), and the result is shown in Figure 3.3. The WT lysate had a band pattern that is characteristic of GP63 detection using this primary antibody (Lane 1). A protein of size 63kDa (GP63) was present; a second protein of 50kDa was also present at a lower abundance. Whether this smaller protein is an isoform, or degradation product of GP63, or whether it is an unrelated cross-reacting protein is unclear. The GPI8 null-mutant lacked GP63 in its cell lysate, though the protein was not completely absent as a trace of protein of size 63kDa could be detected (Lane 2). Re-expression of the native gene restored GP63 (Lane 3). In Δ*gpi8* expressing GPI8^{H174A} or GPI8^{C216G}, GP63 was not detected (lanes 6 and 7 respectively), whereas GP63 was present in cell lines expressing GPI8^{H63A} or GPI8^{C94G} (Lanes 4 and 5). This suggests the mutant forms of GPI8 expressed in the Δ*gpi8*[pX*gpi8*^{H174A}] and Δ*gpi8*[pX*gpi8*^{C216G}] cell lines are not active.

3.2.3.2 Immunofluorescence of surface bound GP63

Surface expression of GP63 was examined by immunofluorescence microscopy. The cells were prepared as described in Chapter 2. Cells were washed in PBS, air dried onto slides, fixed with 2.5% paraformaldehyde/ PBS for 15 minutes, and washed in PBS before the addition of primary antibody diluted as appropriate in PBS. Cells were not treated with methanol/ acetone during the fixation step, therefore cell membranes were not permeabilised. This fixation method detected protein expression on the cell surface. The primary antibody (L3.8) was a mouse monoclonal antibody raised against *L. mexicana* GP63, and was a gift from Dr. David Russell, Washington University, St Louis, USA (Medina-Acosta *et al.*, 1989). L3.8 was used at a dilution of 1:100. The secondary antibody was an anti-mouse FITC conjugate (Sigma), used at a dilution of 1:500. Cells were visualised by fluorescence microscopy and the images are shown in Figure 3.4. WT cells exhibited strong cell surface fluorescence. The secondary antibody only control confirmed that this fluorescence was due to the protein detected by the primary αGP63 antibody, and was surface-bound GP63. Surface bound GP63 was absent from the Δ*gpi8* cell line, but was restored in the Δ*gpi8*[pX*GPI8*] cells. GP63 was also restored to the surface of Δ*gpi8* cells re-expressing the GPI8 mutants GPI8^{H63A} and GPI8^{C94G}, but was absent from the surface of Δ*gpi8* cells re-expressing the GPI8 mutants GPI8^{H174A} and GPI8^{C216G}. These results confirm the data from the western blotting experiments; the GPI8 proteins expressed from the Δ*gpi8*[pX*gpi8*^{H174A}] and

Δ*gpi8*[pX*gpi8*^{C216G}] cell lines are unable to restore GP63 to the cell surface, demonstrating that GPI8^{H174A} and GPI8^{C216G} lack GPIT activity.

3.3 Evidence that GPI8 is a component of a larger complex

In yeast and mammalian cells GPI8 has been shown to form a complex with other proteins to form an active GPIT (Meyer *et al.*, 2000; Vidugiriene *et al.*, 2001). In an attempt to define the role of GPI8 as part of a complex in *L. mexicana*, the gene encoding non-functional GPI8^{C216G} was expressed from an episome in wild type cells (to give cell line WT[pX*gpi8*^{C216G}]). This cell line was compared with wild type promastigotes expressing episomal GPI8 (cell line WT[pX*GPI8*]).

The plasmids pGL269, and pGL403 were transfected into WT cells. The cell lines were grown initially in medium containing 25μg ml^{-1} of the antibiotic G418, to select for the episome. However, to increase the level of GPI8 expression within these cell lines, the concentration of G418 in the culture medium was increased, thereby increasing the copy number of the episome and hence protein expression. Cultures were grown in medium containing 125μg ml^{-1} of G418. Cells from these cultures were added to medium containing 500μg ml^{-1} G418. For each of the two cell lines, cultures were maintained in medium containing the 3 different concentrations of G418.

Expression of GPI8 was verified in the two cell lines WT[pX*gpi8*^{C216G}] and WT[pX*GPI8*], by metabolic labelling of cells grown in 125μg ml^{-1} G418. Cells were grown to mid to late log stage and labelled with ^{35}S-ExpreSS for 6 hours prior to immune-precipitation with an α-GPI8 antibody. Protein samples were electrophoresed by 12% SDS-PAGE, the gel dried and visualised with a Typhoon Phophor Imager (Figure 3.5). A protein of size 42kDa was present in both the WT[pX*gpi8*^{C216G}] and WT[pX*GPI8*] cell lines. This confirms GPI8 episomal expression in these cell lines.

The expression of GPI-anchored GP63 in the two cell lines was examined by western blot analysis (Figure 3.6). WT cells expressing the functional episomal copy of *GPI8* (Lanes 3-5) were found to express GP63 at levels similar to the wild-type parasites (Lane 1). An increase in the concentration of G418, and hence GPI8 levels, did not alter the levels of GP63 (Compare lanes 3, 4 and 5). However, cells expressing the mutated

form of *GPI8* showed decreased levels of GP63 in the cells compared to WT cells (Compare lane 1 with lane 6), and the amount of GP63 decreased as $GPI8^{C216G}$ expression increased (Compare lanes 6, 7 and 8). Expression of an inactive GPI8 in WT cells therefore affects GP63 processing.

The effect on GP63 cell surface expression was then examined by immunofluorescence microscopy (Figure 3.7). Over-expression of GPI8 did not effect GP63 surface expression, whilst expression of $GPI8^{C216G}$ in wild-type cells drastically reduced the amount of GP63 on the cell surface (see cells grown in 25μg ml^{-1} G418). At the highest concentration of antibiotic (500 μg ml^{-1}), surface expression of GP63 was almost undetectable. This demonstrates that expression of $GPI8^{C216G}$ in WT cells inhibits cell-surface anchoring of GP63.

Thus, expression of inactive GPI8 in wild type promastigotes produced a pronounced dominant-negative effect, which provides compelling evidence that GPI8 is required for transamidation activity and is likely to be part of a GPIT complex in *L. mexicana*.

3.4 The fate of GP63 in GPI8 mutant cell lines

It has been established that the mutant forms of GPI8 affect GP63 processing, in that GP63 is lost from the cell lysate and cell surface of those cells which do not have an active GPIT. Studies in *T. brucei* suggest that cells which have a mutation at the GPI anchor addition site show a build-up of proteins destined to be GPI-anchored, within the Golgi (Bangs *et al.*, 1997). Here they are thought to be rapidly degraded, suggesting that GPI anchors may have some involvement in the forward trafficking of some GPI-anchored proteins (McDowell *et al.*, 1998). In *Leishmania*, GP63 mutated at the GPI-anchor addition site and expressed in a GP63 deficient cell line, was demonstrated to be secreted from the cell (McGwire and Chang, 1996). Both of these studies in trypanosomatids used artificial reporter systems with a defect at the site of GPI anchor addition. I therefore examined the processing of a GPI-anchored protein in a cell line with a defect in the GPI-anchoring pathway. The processing of GP63 was therefore examined in the cell lines expressing the different mutants of GPI8.

3.4.1 Metabolic labelling of GP63 in different GPI8 mutant cell lines

The fate of GP63 within these cell lines was examined by metabolic labelling. Cells were labelled for 6 hours with [^{35}S]ExpreSS, and GP63 immune-precipitated with the L3.8 antibody from either cell lysate, or the culture supernatants, as described in detail in section 2.5.10. Protein samples were electrophoresed on 12% agarose gels, which were dried down and then exposed to X-Ray film, or the Typhoon Phosphor imager.

Secretion of GP63 was first examined (Figure 3.8, panel A). From WT parasites (Lane 1), a small amount of 3 isoforms of GP63 were detected (63, 64, 65 kDa - designated 63s, 64s and 65s for secreted), with the 63s isoform being the most abundant. In contrast, a large amount of the 65s isoform was detected in the medium in which Δ*gpi8* cells had been grown (Lane 2). Re-expression of *GPI8* in the Δ*gpi8* null mutant resulted in only small amounts of GP63 being secreted, with isoform sizes comparable with those secreted from WT cells (Lane 3). Large quantities of the 65s isoform were detected also in the Δ*gpi8* cell lines expressing GPI8^{C216G} (Lane 7) and GPI8^{H174A} (Lane 6), as well as GPI8^{C94G} (Lane 5). However, the Δ*gpi8* cell line expressing GPI8^{H63A} mimicked the situation in wild type cells with little GP63 secreted. Thus a high level of secretion was associated with cells having a non-functional GPIT. WT cells re-expressing GPI8 or the inactive GPI8^{C216G} from an episome were also examined (Lanes 8 and 9). The cells were grown in 125μg ml^{-1} G418 in order to increase the level of expression from the episome. WT cells over-expressing the functional copy of GPI8, processed GP63 as found in WT cells. A small amount of the 65s, 64s and 63s forms were secreted into the medium, with the 63s being the most abundant. In contrast WT cells over-expressing GPI8^{C216G} process GP63 as found in the Δ*gpi8* cells. A large amount of the 65s isoform of GP63 was secreted into the medium.

The presence of GP63 was also analysed in the cell lysates (Figure 3.5, panel B) by immune-precipitation with the same α-GP63 antibody. Three isoforms of GP63 were detected in WT cells (63, 64, 65 kDa - designated 63c, 64c and 65c for cell-associated), with the 63c isoform being most abundant. In the Δ*gpi8* cell line, only the 65c isoform was detected (Lane 2). The two smaller isoforms of GP63 (63c and 64c) were present in Δ*gpi8*[pX*GPI8*] lysates (Lane 3), at approximately equal levels. Δ*gpi8* cell lines expressing GPI8^{H174A} (Lane 6) or GPI8^{C216G} (Lane 7) were similar in cell-associated

profiles to Δ*gpi8* with the 65c isoform predominating, whereas the Δ*gpi8* cell line expressing GPI8^{H63A} (Lane 4) was the same as wild type with abundant 63c isoform. The Δ*gpi8* cell line expressing GPI8^{C94G} (Lane 5) gave an intermediate pattern, with all three isoforms of GP63 present, however the 65c form was the most prevalent. The pattern in WT cell-lysates over-expressing the functional form of GPI8 was as that found in WT cells only. The pattern in WT cells over-expressing GPI8^{C216G} corresponded to that found in the Δ*gpi8* cell line.

These results confirm that GPI8^{H174A} and GPI8^{C216G} are inactive forms of GPI8, the phenotype of these cell lines mimics that of the Δ*gpi8* cell line. GP63 is secreted into the medium in the cell lines without an active form of GPI8, and is not degraded intracellularly as previously suggested (Hilley *et al.*, 2000).

3.5 Discussion

Re-expression of episomal GPI8 in the Δ*gpi8* cell line provides an excellent model for assessing GPI8 activity. Whilst the activity of the GPIT enzyme cannot be measured directly, GP63 provides a convenient marker for the study of GPI8 activity. The use of the Δ*gpi8* cell line in previous work demonstrated that loss of GPI8 resulted in the loss of GP63 from the cell surface, whilst the episome pXGPI8 was able to rescue this phenotype (Hilley *et al.*, 2000). [^{3}H]ethanolamine labelling confirmed that the Δ*gpi8* cell line was deficient in GPI-anchored GP63, and showed an accumulation of putative protein anchor precursors (Hilley *et al.*, 2000). The episome therefore provides an ideal system for studying the mutation of GPI8.

Precipitation of GPI8 from all cell lines with an episomal copy of GPI8, confirmed that the protein was expressed, and demonstrated that lack of GPI8 activity was not due simply to lack of GPI8 expression. Use of the GPI8-GFP construct provides an important tool in the validation of the R492 GPI8 antibody, as recognition of the predicted 75kDa protein by both αGFP and αGPI8 confirmed that the GPI8 antibody does indeed recognise GPI8. This is an important result, because in previous studies the antibody was unable to recognise *L. mexicana* GPI8 by western blotting, and therefore evidence for the episomal expression of GPI8 was not provided (Hilley, 1999). GPI8

was detected in all cell lines expressing an episomal copy of GPI8 including the Δ*gpi8*[pX*gpi8*^{C216G}] cell line produced in a previous study.

In WT cells GPI8 was not detected (Figure 3.2). This may be due to the natural low level of expression of GPI8 in the WT cells and suggests that all components of GPIT may be expressed at a similarly low level. Use of the episome pX*GPI8* caused vast over expression of GPI8 and related mutants. However, this over expression did not appear to effect GPIT activity as substantiated by the use of both the Δ*gpi8*[pX*GPI8*] and WT[pX*GPI8*] cell lines. In these cell lines GP63 was still processed as found in WT cells.

The GPI8 mutations GPI8^{H163A} and GPI8^{C94G} restored GPIT activity, as assessed by western blotting and immunofluorescence microscopy of GP63. However, the mutations GPI8^{H174A} and GPI8^{C216G} were not able to restore GP63 to the cell surface, indicating that these two mutant forms of GPI8 are inactive and unable to transfer a GPI anchor to the C-terminus of GP63. This provides evidence that the residues H174 and C216 of GPI8 are essential for GPI anchoring, and indicates that these are the active site residues of *L. mexicana* GPI8.

It is possible that the mutations GPI8^{H174A} and GPI8^{C216G} each cause conformational changes in the GPI8 protein, which inactivates the protein. This is a possibility as the structure of the protein is at present not known. The importance of a single residue in the maintenance of tertiary structure is feasible in the case of cysteine, as it is possible that the sulphydryl group interacts with a second cysteine to form a disulfide bridge. It is possible that the residue C216 interacts with one of the other 8 cysteine residues present in *L. mexicana* GPI8. However cysteine residues involved in disulfide bridge formation would be expected to be conserved amongst the GPI8 subfamily. Mutation of the only other conserved residue, C94, does not abolish GPIT activity. Expression of GPI8^{C216G} in WT cells also suggests that loss of activity is due to the loss of the catalytic site rather than a conformational change. It seems unlikely that a protein inactivated due to conformational changes would be able to interact with other complex members, and therefore could not out compete the resident WT protein, producing a dominant negative effect. Comparison of this work with other studies provides further

evidence that H174 and C216 are not responsible for maintenance of protein structure, but are the catalytic dyad for GPI8.

The identification of C216 as a catalytically important residue complements results which demonstrate that a cysteine group acts as the active site residue for the *T. brucei* GPIT (Sharma *et al.*, 1999b), and *L. mexicana* GPI8 (Sharma *et al.*, 2000). A cell-free system was established to examine the mechanism of GPI anchor addition and utilised trypanosome membranes with endogenous GPIT activity and detectable amounts of the GPI-anchored protein VSG. The small nucleophile hydrazine was used as a nucleophile substitute for the ethanolamine residue of the GPI anchor. Addition of sulfhydryl alkylating agents into the assay resulted in the inhibition of hydrazine-induced release of VSG, and this was concluded to be due to the inactivation of an active site cysteine (Sharma *et al.*, 1999b). In a similar experiment using a mammalian system, the sulfhydryl alkylating agents did not reduce the amount of preprominiPLAP converted to GPI-anchored miniPLAP (Sharma *et al.*, 1999b). However, this was thought to be due to a limitation of the assay, rather than to a difference in activity between trypanosome and mammalian GPIT. The mammalian system used an *in vitro* translated reporter protein, which was added to membranes. Sulfhydryl alkylating agents were not added at the outset of the assay as they inhibit translocation, and it was thought GPI anchor addition had occurred during this delay (Sharma *et al.*, 1999b). Washed trypanosome membranes lacked GPIT activity, and this deficiency was reversed by the addition of recombinant *L. mexicana* GPI8 to the assay (Sharma *et al.*, 2000), whilst recombinant GPI8 incubated first with sulfhydryl alkylating agents could not reconstitute activity. These data provide evidence that the active site residue of *T. brucei* and *L. mexicana* GPI8 is a cysteine.

Sequence analysis has demonstrated that GPI8 has identity to the Clan CD, C13 family of cysteine proteases (Benghezal *et al.*, 1996; Hilley *et al.*, 2000). Legumain from the plant legume, the jack bean, *Canavalia ensiformis*, was the first protein in this C13 family to be identified. It showed specificity for the cleavage of asparaginyl bonds, and was inhibited by sulfhydryl alkylating agents (Abe *et al.*, 1993; Barrett and Rawlings, 1996). Legumain-like proteins have since been identified and characterised in mammals including mouse (Chen *et al.*, 1997) and pig (Chen *et al.*, 1998). Families of

cysteine peptidases are characterised by highly conserved regions which form the catalytic site (Barrett and Rawlings, 1996). Within the C13 family this consists of a catalytic dyad; a histidine residue is thought to deprotonate the active site cysteine (Barrett and Rawlings, 1996; Meyer *et al.*, 2000). The GPI8 homologues appear to form a subfamily of the original C13 family (Meyer *et al.*, 2000). The asparaginyl endopeptidase subfamily shows significant sequence similarity around the active site histidine/ cysteine, whilst the transamidase subfamily shows little homology around the active site histidine/ cysteine (Sajid and McKerrow, 2002).

The active site residues of yeast GPI8 (Meyer *et al.*, 2000), and mammalian GPI8 (Ohishi *et al.*, 2000) have recently been identified. Sequence alignment of the C13 and GPI8 families identified in yeast Cys85 as conserved amongst the GPI8 family, whilst C199 was conserved amongst the entire C13/ GPI8 families with the exception of *Schistosoma mansoni* hemoglobinase B (Meyer *et al.*, 2000). His94 was conserved in 18 of the 19 identified C13/ GPI8 family members, whilst H157 was conserved in all 19. Deletion of GPI8 from yeast cells is lethal, therefore the four conserved sites were mutated and used in complementation experiments in a yeast GPI8/ Δgpi8 mutant and WT cells. The Cys199 and His157 mutations were unable to rescue Δgpi8 spores in tetrad analysis, whilst expression in WT cells led to growth arrest, and the build up of GPI anchor precursors. Thus, C199 and H157 were identified as the active site residues of yeast GPI8 (Meyer *et al.*, 2000). A similar approach identified Cys206 and His164 as the active site residues in human GPI8 (Ohishi *et al.*, 2000). C206A and H164A expressing vectors were unable to complement K-cells, a mammalian cell line which lack GPIT activity. The addition of C206A and H164A GPI8 to K-cell membranes failed to produce the hydrazide form of miniPLAP in a cell free assay, demonstrating the absence of GPIT activity (Ohishi *et al.*, 2000). The residues H174 and C216 of *L. mexicana* GPI8 are homologous to those identified as the active site histidine and cysteine residues in yeast and human GPI8s (Figure 3.1). The data presented provides convincing evidence that H174 and C216 are the *L. mexicana* GPI8 catalytic dyad.

Metabolic labelling of cells and immune-precipitation of GP63 reveals that while the mutant GPI8^{C94G} does not abolish GPIT activity, it does, however, show an aberrant phenotype when compared with WT cells. Similar to WT, GP63 is trafficked to the cell

surface, as demonstrated by immuno-fluorescence microscopy, and processed to a 63 kDa form as demonstrated by metabolic labelling and immune-precipitation with αGP63. However, unlike WT cells, the Δ*gpi8*[pX*gpi8*^{C94G}] cell line secretes a large amount of a 65kDA form of GP63 into the medium. The 65s form of GP63 is abundantly secreted from Δ*gpi8* cells and the cell lines expressing the active site mutants GPI8^{H174A} and GPI8^{C216G}, indicating this phenotype is associated with an inactive GPIT and is the result of the failure to add a GPI anchor to the GP63 protein. GPI8^{C94G} is therefore a dysfunctional enzyme, with reduced GPIT activity (Figure 3.9). Mutation of His54 in yeast GPI8 and Cys92 in human GPI8 also led to a partial loss in function (Meyer *et al.*, 2000; Ohishi *et al.*, 2000). It is not clear if this partial loss in function is due to improper folding of the proteins, possibly preventing efficient integration into a GPIT complex. The residues may have some catalytic importance, or play a role such as the recruitment of GP63 into the GPIT complex. Alternatively, the residue may be implicated in linking GPI8 to other complex members. As C94 is conserved amongst all the GPI8 family, but not the C13 subfamily (See Figure 3.1), it is possible that this residue forms an intercomplex disulfide bridge with another member of the putative GPIT complex. This is possible particularly as the homologous mutation in human GPI8 (C92A) causes a similar decrease in activity (Ohishi *et al.*, 2000). The corresponding mutant in yeast (C85A), was only assessed for its ability to complement the GPI8 deletion, and the level of GPI8 activity in this mutant was not addressed (Meyer *et al.*, 2000).

Expression of the active site mutant GPI8^{C216G} in WT cells leads to a pronounced dominant negative effect. GP63 was not processed to the cell surface, and was secreted from the cells in the 65s form, similar to the phenotype seen in Δ*gpi8* cells. This was not an artefact caused by the over-expression of GPI8 from an episome, as demonstrated by the finding that expression of wild type GPI8 from an episome did not effect GP63 processing. This provides evidence that GPI8 is a component of a larger GPIT complex. GPI8 was undetectable in WT cells by immune-precipitation, so is likely to be expressed in the cell at a low level. Other complex members would be expected to be expressed at comparable levels, competition between functional and non-functional GPI8 to form this GPIT complex would be limiting. It could be argued that the vast over-expression of GPI8^{C216G} results in the mutant form of GPI8 out-

competing the WT form only with regard to the proteins binding to GP63. However, competition to bind to GP63 is unlikely to be limiting as GP63 is an abundant protein in the cell, making up approximately 1% of the total protein in promastigotes (Bouvier *et al.*, 1995).

The dynamics of this dominant negative effect could not be examined by the method used. Expression of GPI8 from the pXG episome could not be switched on or off, nor the level of GPI8 expression be tightly controlled, therefore the rapidity with which the GPIT reaction was inhibited could not be assessed. Use of an inducible expression system would allow a closer examination of the kinetics of GPIT complex formation, both with respect to timing, and the level of expression of $GPI8^{C216G}$ required to produce a dominant negative effect.

Evidence from other systems also demonstrates that GPI8 is the catalytic sub-unit within a larger GPIT complex. Removal of *T. brucei* GPIT activity from trypanosome membranes by a high pH wash, and reconstitution of that activity with LmGPI8-his, suggested that in protozoa the GPIT is part of a complex, and this complex may be dynamic (Sharma *et al.*, 2000). *L. mexicana* and *T. brucei* GPI8 lack a transmembrane domain, and it is conceived that at least one other component with a transmembrane domain is present in the complex to link GPI8 to the ER membrane. Binding of pro-protein to the GPIT has been shown by the cell-free assay system to be reversible (Sharma *et al.*, 1999b).

In higher eukaryotes GPI8 has been demonstrated to form part of a complex with at least three other components (GAA1, GPI16/ PIG-S, and GPI17/ PIG-T), which associate stably (Ohishi *et al.*, 2000; Fraering *et al.*, 2001; Ohishi *et al.*, 2001). In yeast cells, GAA1 and GPI8 were first cloned from a temperature-sensitive GPI deficient cell line (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996). Immune-precipitation experiments demonstrated that GAA1 and GPI8 associate as a complex, with a third protein GPI16 (Fraering *et al.*, 2001). Yeast GPI17 was identified through homology with the mammalian protein PIG-T (Ohishi *et al.*, 2001). In mammalian cells homologues of yeast GPI8 and GAA1 were identified, (Yu *et al.*, 1997; Hiroi *et al.*, 1998), and demonstrated to act as components of a GPIT complex (Ohishi *et al.*, 2000). PIG-S and PIG-T were isolated as GPIT complex members by co-precipitation with

GPI8. The 4 known members of the GPIT complex were each shown to be essential for GPI anchor attachment (Ohishi *et al.*, 2000; Fraering *et al.*, 2001).

Studies using photo-crosslinking methods were able to co-precipitate GPI8, GAA1 and 2 additional proteins of sizes 60 and 120 kDa from mammalian cells (Vidugiriene *et al.*, 2001). The 120kDa protein does not match the size of any components of the GPIT complex so far identified. The GPI complex was also shown to sediment at a size corresponding to 460kDa, and not in the range for 240kDa, which is the size, predicted from the 4 known components (Vainauskas *et al.*, 2002). This suggests other members of the GPIT complex may exist.

GPI8 can be cross-linked directly to the pro-protein, demonstrating a direct association occurs between GPI8 and the protein to be anchored (Spurway *et al.*, 2001; Vidugiriene *et al.*, 2001). This provides further evidence that GPI8 is the catalytic sub-unit of the GPIT complex, and directly cleaves the GPI attachment signal peptide. The role of the other complex members is unclear. GAA1 has 6 transmembrane domains and is thought to anchor the catalytic subunit to the ER membrane. It is anticipated that some GPI-attachment signal-peptide recognition mechanism exists to prevent cleavage of unrelated proteins, and it is speculated that other sub-units of the complex may regulate this (Meyer *et al.*, 2000). A 70 kDa protein, possibly GAA1, could be cross-linked to the pro-protein, but only in conditions whereby the protein was unable to be GPI-anchored. This suggests that GAA1 may directly associate with the pro-protein and have a functional role within the GPIT complex (Vidugiriene *et al.*, 2001). However, use of the cysteine-specific cross-linking reagent BMH detected pro-protein, with a mutation at the ω site, linked to GPI8, but not GAA1. It was speculated that this may be due to lack of interaction between GAA1 and the pro-protein, or that the method used was not sensitive enough to detect the interaction (Spurway *et al.*, 2001). Loss of PIG-T prevents the complex formation of the other three members, suggesting a critical role for PIG-T in the maintenance of the complex (Ohishi *et al.*, 2001).

It is unclear if the GPIT complex formed in higher eukaryotes is stable or dynamic. The dynamics of the dominant negative effect produced by expression of a GPI8 active site mutant in yeast cells suggested rapid incorporation of the mutated protein into existing complexes. This implies that the GPIT complex is dynamic in nature (Meyer *et al.*,

2000). However, it is clear that the complex is relatively stable as the sub-units do not dissociate during extraction and purification, even in the absence of pro-protein and GPI anchor precursors (Ohishi *et al.*, 2001).

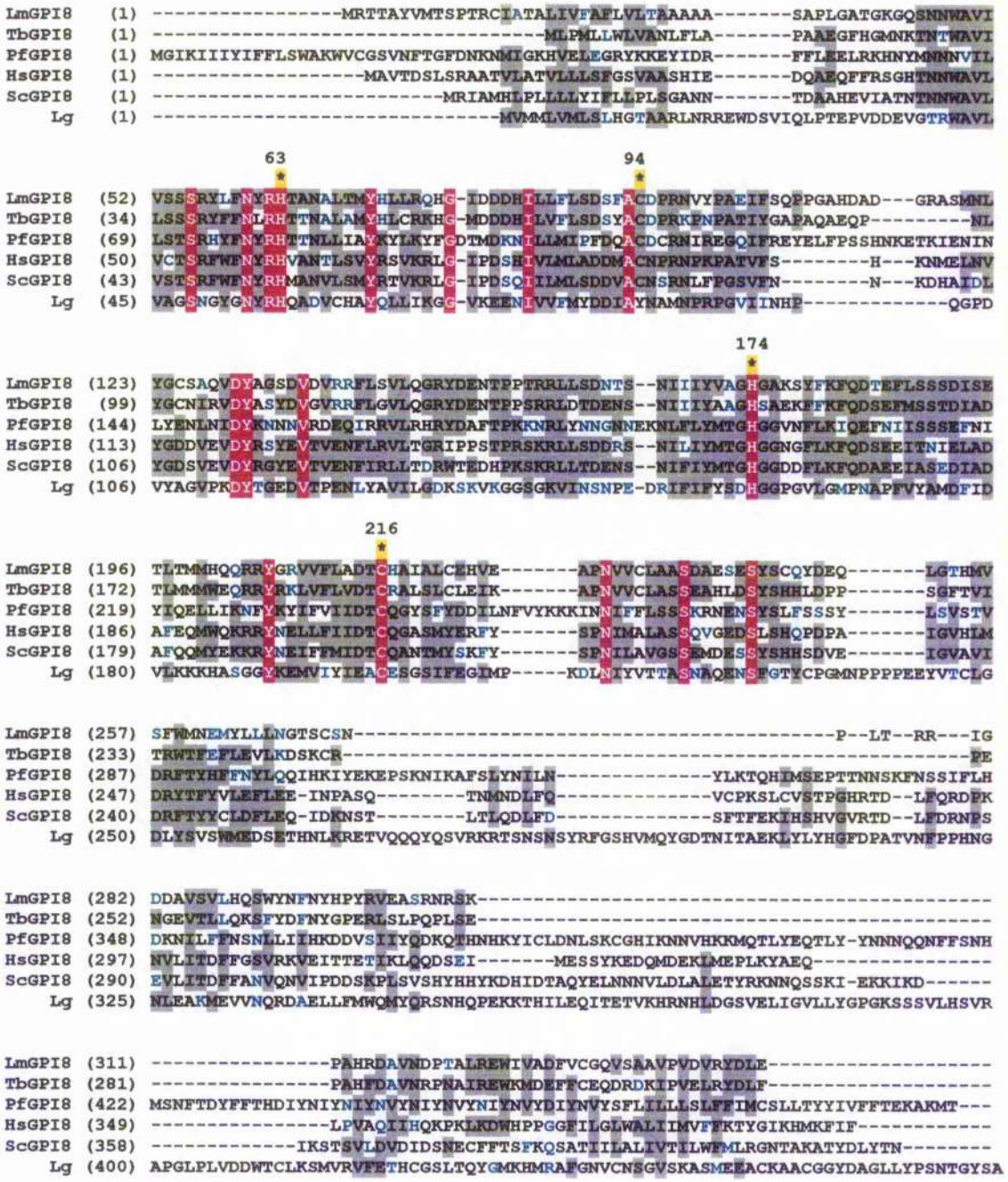
The GPI: Protein transamidase has been demonstrated in higher eukaryotes to be a large complex containing at least 4 members. While no other members of the GPIT complex have yet been cloned from protozoa, sequence analysis has identified a putative GAA1 homologue in *L. major* (Eisenhaber *et al.*, 2001). The data presented strongly indicates *L. mexicana* GPI8 is the catalytic sub-unit of a larger GPIT complex, reminiscent of the situation found in higher eukaryotes. Identification of other complex members, and the effects of the loss of GPI anchoring on the onward processing and trafficking of GPI-anchored proteins, represent areas for further study.

The fate of GP63 was examined in the cell lines with an active, inactive and dysfunctional GPI8. GP63 was secreted at high levels into the medium in the absence of GPI8 activity, as demonstrated by metabolic labelling and immune-precipitation analysis (Figure 3.8). This is in contrast with previously reported findings, where GP63 was not detectable in the culture medium by western blotting, and was thought to be degraded intracellularly (Hilley, 1999; Hilley *et al.*, 2000). This is an indicator of the greater sensitivity of the use of immune-precipitation and metabolic labelling. The results demonstrate that GP63 is processed and trafficked differently in cells that lack a functional GPIT (Δ*gpi8*, Δ*gpi8*[pX*gpi8*^{C216G}], Δ*gpi8*[pX*gpi8*^{H174A}], WT[pX*gpi8*^{C216G}]). Failure to add a GPI anchor to the protein results in secretion of GP63 from the cell instead of surface anchoring, and this secreted isoform is of size 65kDa. Proteins with a functional GPIT produce GPI-anchored GP63, which is trafficked to the cell surface, and has isoforms of 3 different sizes (63kDa, 64Da and 65kDa). These isoforms may represent GP63 at different stages of processing. The presence of different subsets of GP63 isoforms within the 2 sets of cell lines may be due to differences in processing, such as the failure to remove the C-terminal signal sequence in cell lines lacking GPIT activity. The processing of GP63 will be addressed in the next chapter.

GPI8 activity can be defined with respect to the fate of GP63. These results are summarised in Figure 3.9. Cells with an active GPI8 traffic GP63 to the cell surface, with a small amount secreted. Cells with an inactive GPI8 do not traffic GP63 to the

cell surface, and secrete an abundant amount of GP63 into the medium. $GPI8^{C216G}$ and $GPI8^{H174A}$ are non-functional enzymes, whereas $GPI8^{H63A}$ is fully functional. The cell line expressing dysfunctional $GPI8^{C94G}$ has an intermediate phenotype, with GP63 trafficked to the cell surface, but also a large quantity of GP63 secreted from the cell.

Figure 3.1: Amino acid sequence alignment of *Leishmania mexicana* GPI8 with homologues from other species, and Legumain a C13 cysteine protease.
L. mexicana (Lm) GPI8 (Acc No:AJ242865/ Protein ID:CAB55340) alignment with GPI8 from *T. brucei* (Tb (AJ439686/ CAD291141)), *Plasmodium falciparum* (Pf (AJ401202/ CAD96076)), *Saccharomyces cerevisiae* (Sc (AQ450372/ P49018)) and *Homo sapiens* (Hs (AF022913/ AAB81597)), and *Canavalia ensiformis* Legumain (Lg (D31787/ BAA06599.1)) a member of the C13 cysteine protease sub-family. The alignment was performed using Align X (InforMax. Inc). Identical residues are shaded pink, conserved and similar residues are shaded grey. Weakly similar residues are shown in blue. Conserved cysteine and histidine residues which are potential active site residues are indicated (*), and numbered according to their position within the *L. mexicana* GPI8 sequence.


LmGPI8 (1) ----------MRTTAYVMTSPTRCIATALIVFAFLVLTAAAAA-------SAPLGATGKGQSNNWAVI
TbGPI8 (1) ----------MLPMLLWLVANLFLA-------PAAEGFHGMNKTNTWAVI
PfGPI8 (1) MGIKIIIYIFFLSWAKWVCGSVNFTGFDNKNMIGKHVELEGRYKKEYIDR-------FFLEELRKHNYMNNNVIL
HsGPI8 (1) ----------MAVTDSLSRAATVLATVLLLSFGSVAASHIE-------DQAEQFFRSGHTNNWAVL
ScGPI8 (1) ----------MRIAMHLPLLLLYIFLLPLSGANN-------TDAAHEVIATNTNNWAVL
Lg (1) ----------MVMMLVMLSLHGTAARLNRREWDSVIQLPTEPVDDEVGTRWAVL
63
94
LmGPI8 (52) VSSSRYLFNYRHTANALTMYHLLRQHG-IDDDHILLFLSDSFACDPRNVYPAEIFSQPPGAHDAD---GRASMNL
TbGPI8 (34) LSSSRYFFNIRHTTNALAMYHLCRKHG-MDDDHILVFLSDSYACDPRKPNPATIYGAPAQAEQP---------NL
PfGPI8 (69) LSTSRHYFNYRHTTNLLIAYKYLKYFGDTMDKNILLMIPFDQACDCRNIREGQIFREYELFPSSHNKETKIENIN
HsGPI8 (50) VCTSRFWFNYRHVANTLSVYRSVKRLG-IPDSHIVLMLADDMACNPRNPKPATVFS--------H---KNMELNV
ScGPI8 (43) VSTSRFWFNYRHMANVLSMYRTVKRLG-IPDSQIIIMLSDDVACNSRNLFPGSVFN--------N---KDHAIDL
Lg (45) VAGSNGYGNYRHQADVCHAYQLLIKGG-VKEENIVVFMYDDIAYNAMNPRPGVIINHP----------QGPD
174
LmGPI8 (123) YGCSAQVDYAGSDVDVRRFLSVLQGRYDENTPPTRRLLSDNTS--NIIIYVAGHGAKSYFKFQDTEFLSSSDISE
TbGPI8 (99) YGCNIRVDYASYDVGVRRFLGVLQGRYDENTPPSRRLDTDENS--NIIIYAAGHSAEKFFKFQDSEFMSSTDIAD
PfGPI8 (144) LYENLNIDYKNNNVRDEQIRRVLRHRYDAFTPKKNRLYNNGNNEKNLFLYMTGHGGVNFLKIQEFNIISSSEFNI
HsGPI8 (113) YGDDVEVDYRSYEVTVENFLRVLTGRIPPSTPRSKRLLSDDRS--NILIYMTGHGGNGFLKFQDSEEITNIELAD
ScGPI8 (106) YGDSVEVDYRGYEVTVENFIRLLTDRWTEDHPKSKRLLTDENS--NIFIYMTGHGGDDFLKFQDAEEIASEDIAD
Lg (106) VYAGVPKDYTGEDVTPENLYAVILGDKSKVKGGSGKVINSNPE-DRIFIFYSDHGGPGVLGMPNAPFVYAMDFID
216
LmGPI8 (196) TLTMMHQQRRYGRVVFLADTCHAIALCEHVE-------APNVVCLAASDAESESYSCQYDEQ-------LGTHMV
TbGPI8 (172) TLMMMWEQRRYRKLVFLVDTCRALSLCLEIK-------AENVVCLASSEAHLDSYSHHLDPP-------SGFTVI
PfGPI8 (219) YIQELLIKNEYKYIFVIIDTCQGYSFYDDILNFVYKKKINNIFFLSSSKRNENSYSLFSSSY-------LSVSTV
HsGPI8 (186) AFEQMWQKRRYNELLFIIDTCQGASMYERFY-------SPNIMALASSQVGEDSLSHQPDPA-------IGVHLM
ScGPI8 (179) AFQQMYEKKRYNEIFFMIDTCQANTMYSKFY-------SENILAVGSSEMDESSYSHHSDVE-------IGVAVI
Lg (180) VLKKKHASGGYKEMVIYIEACESGSIFEGIMP-----KDLNIYVTTASNAQENSFGTYCPGMNPPPPEEYVTCLG
LmGPI8 (257) SFWMNEMYLLLNGTSCSN----------P--LT--RR---IG
TbGPI8 (233) TRWTFEFLEVLKDSKCR----------PE
PfGPI8 (287) DRFTYHFFNYLQQIHKIYEKEPSKNIKAFSLYNILN----------YLKTQHIMSEPTTNNSKFNSSIFLH
HsGPI8 (247) DRYTFYVLEFLEE-INPASQ--------TNMNDLFQ----------VCPKSLCVSTPGHRTD--LFQRDPK
ScGPI8 (240) DRFTYYCLDFLEQ-IDKNST--------LTLQDLFD----------SFTFEKIHSHVGVRTD--LFDRNPS
Lg (250) DLYSVSWMEDSETHNLKRETVQQQYQSVRKRTSNSNSYRFGSHVMQYGDTNITAEKLYLYHGFDPATVNFPPHNG
LmGPI8 (282) DDAVSVLHQSWYNFNYHPYRVEASRNRSK----------
TbGPI8 (252) NGEVTLLQKSFYDFNYGPERLSLPQPLSE----------
PfGPI8 (348) DKNILFFNSNLLIIHKDDVSIIYQDKQTHNHKYICLDNLSKCGHIKNNVHKKMQTLYEQTLY-YNNNQQNFFSNH
HsGPI8 (297) NVLITDFFGSVRKVEITTETIKLQQDSEI-------MESSYKEDQMDEKLMEPLKYAEQ----------
ScGPI8 (290) EVLITDFFANVQNVIPDDSKPLSVSHYHHYKDHIDTAQYELNNNVLDLALETYRKNNQSSKI-EKKIKD------
Lg (325) NLEAKMEVVNQRDAELLFMWQMYQRSNHQPEKKTHILEQITETVKHRNHLDGSVELIGVLLYGPGKSSSVLHSVR
LmGPI8 (311) ----------PAHRDAVNDPTALREWIVADFVCGQVSAAVPVDVRYDLE----------
TbGPI8 (281) ----------PAHFDAVNRPNAIREWKMDEFTCEQDRDKIPVELRYDLF----------
PfGPI8 (422) MSNFTDYFFTHDIYNIYNIYNVYNIYNVYNIYNVYDIYNVYSFLILLLSLFFIMCSLLTYYIVFFTEKAKMT---
HsGPI8 (349) ----------LPVAQIIHQKPKLKDWHPPGGFILGLWALIIMVFFKTYGIKHMKFIF----------
ScGPI8 (358) ----------IKSTSVLDVDIDSNECFFTSFKQSATIILALIVTILWFMLRGNTAKATYDLYTN--------
Lg (400) APGLPLVDDWTCLKSMVRVFETHCGSLTQYGMKHMRAFGNVCNSGVSKASMEEACKAACGGYDAGLLYPSNTGYSA

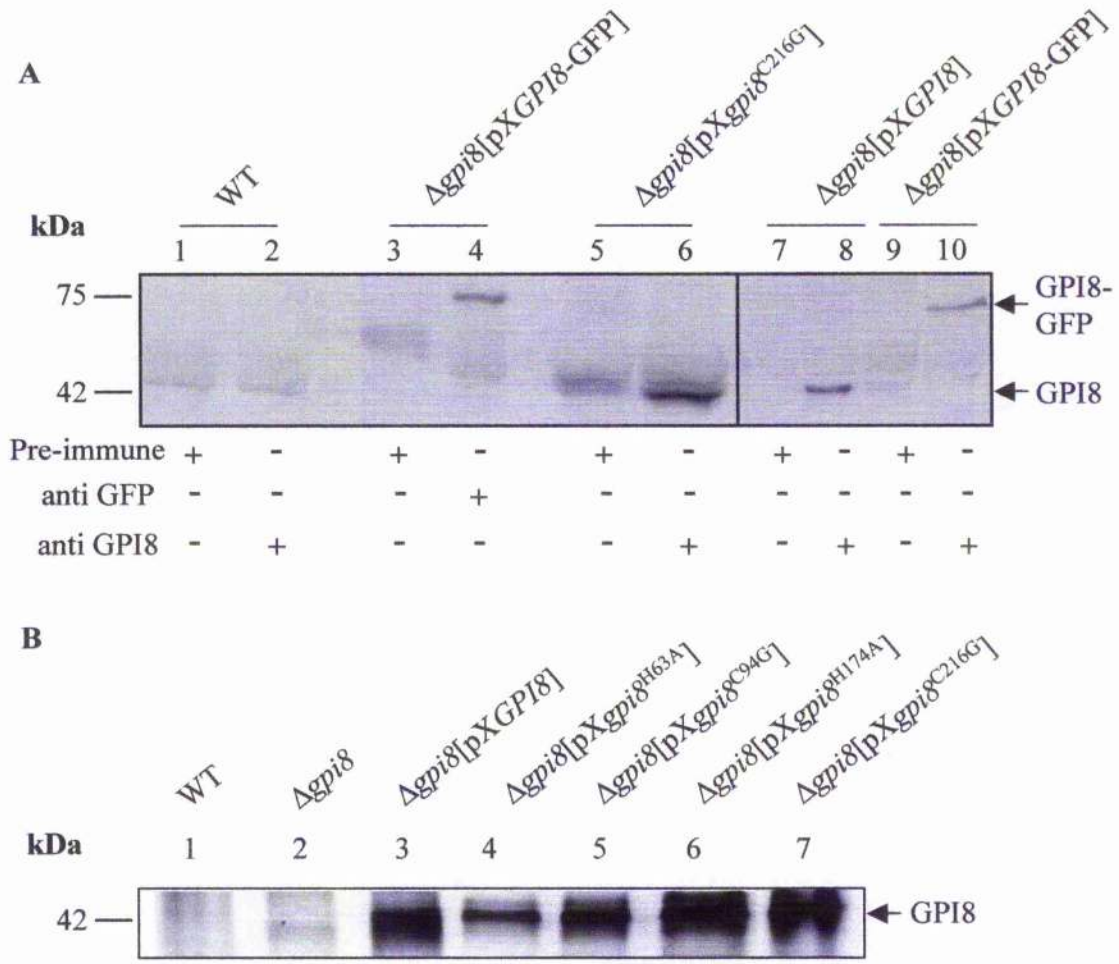


Figure 3.2: Analysis of GPI8 expression in cell lines containing an episome. Cell lines were metabolically labelled with S^{35}ExpreSS for 6 hours hours prior to immune-precipitation from the cell lysate. Equivalent samples were electrophoresed by 12% SDS PAGE, the gels dried down and visualised with a Typhoon Phosphor imager.

Panel A) Samples were immune-precipitated with the R492 GPI8 antibody (Lanes 2, 6, 8 and 10), or a GFP antibody (Lane 4). Alternatively samples were immune-precipitated with R492 pre-immune serum (Lanes1, 3, 5, 7 and 9), to demonstrate antibody specificity. The 42kDa protein GPI8, and the 72kDa protein GPI8-GFP are indicated

Panel B) Samples were immune-precipitated with the R492 GPI8 antibody, to confirm GPI8 expression in the Δ*gpi8* cell lines re-expressing different forms of GPI8 from an episome.

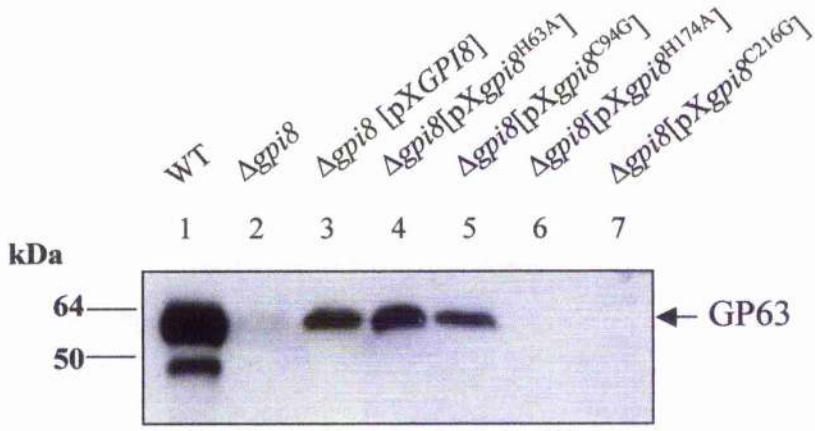


Figure 3.3: Analysis of GP63 expression in cell lines expressing modified GPI8. Cell lysates were prepared from the cell lines WT, Δ*gpi8,* Δ*gpi8*[pX*GPI8*], Δ*gpi8*[pX*gpi8*^{H63A}], Δ*gpi8*[pX*gpi8*^{C94G}], Δ*gpi8*[pX*gpi8*^{H174A}] and Δ*gpi8*[pX*gpi8*^{C216G}]. These were subjected to SDS-PAGE and electrophoresed at $1x10^7$ cell equivalents per lane, and electroblotted. The blot was exposed to a *L. major* GP63 monoclonal antibody at a dilution of 1:50. The 63kDa GP63 protein is indicated.

Figure 3.4: Immunofluorescence detection of surface bound GP63.

L. mexicana promastigotes were fixed in 2.5% paraformaldehyde and cells were incubated with a GP63 primary antibody (L3.8) at a dilution of 1:100, and a FITC conjugated anti-mouse secondary antibody (Sigma) at a dilution of 1:500.

Cells were examined with a Zeiss microscope and images were visualised using a Hamamatsu digital camera and analysed in OpenLab.

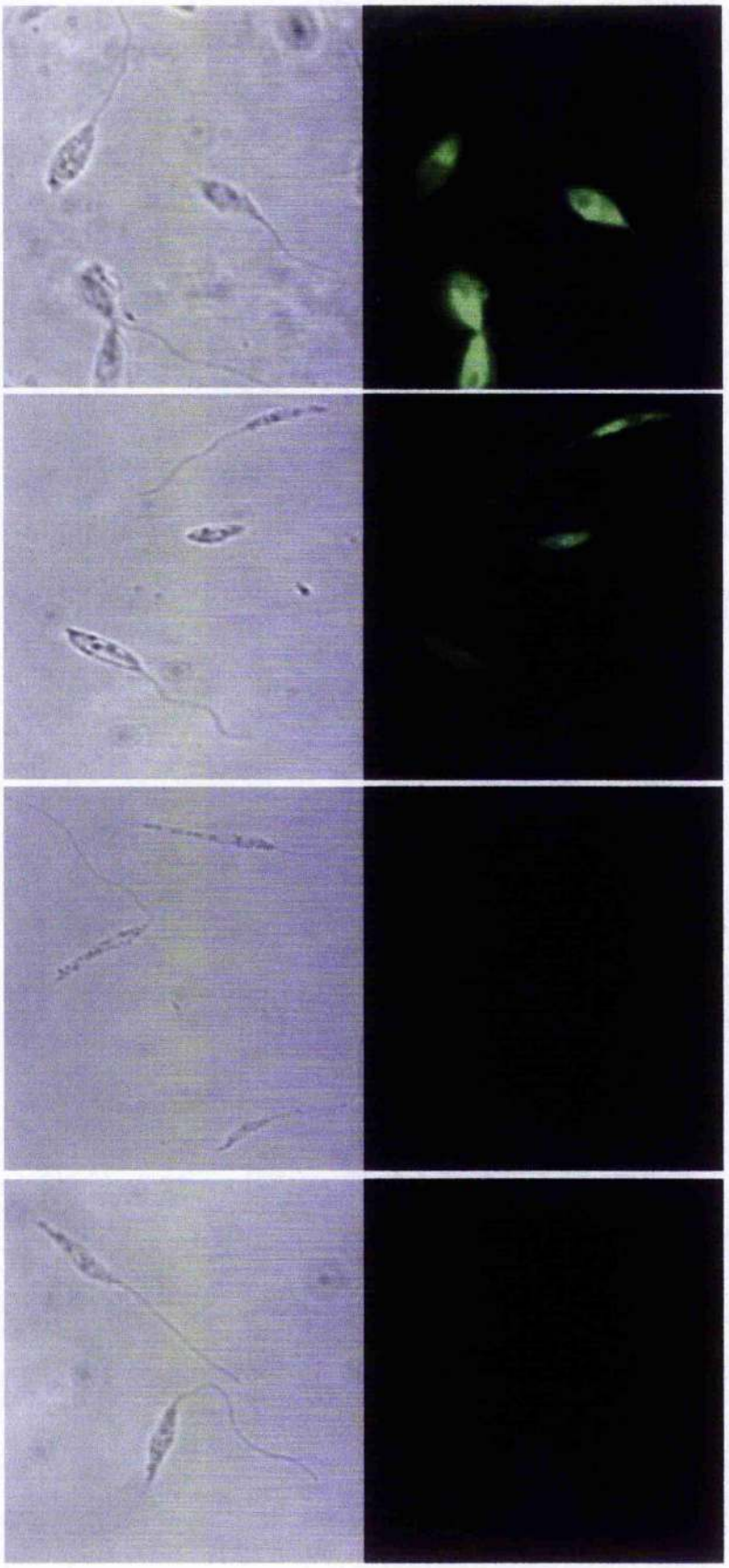
WT

Δ*gpi8*

Δ*gpi8*[pX*GPI8*]

WT
2' antibody only

Phase **Fluorescence**


Δgpi8[pXgpi8H63A]
Δgpi8[pXgpi8C94G]
Δgpi8[pXgpi8H174A]
Δgpi8[pXgpi8C216G]
Phase
Fluorescence

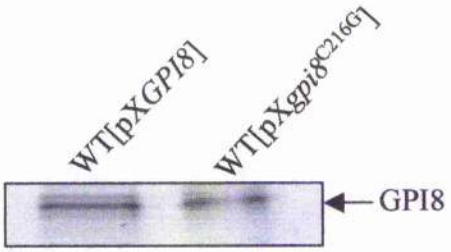


Figure 3.5: Expression of GPI8 in WT promastigotes expressing episomal copies of *GPI8*.

Cell lines were grown in 125 μg ml^{-1} G418, and metabolically labelled with S^{35}ExpreSS for 6 hours hours prior to immune-precipitation from the cell lysates with the R492 α-GPI8 polyclonal antibody. Equivalent samples were electrophoresed by 12% SDS PAGE, the gel dried down and visualised with a Typhoon Phosphor imager. The 42kDa GPI8 protein is indicated.

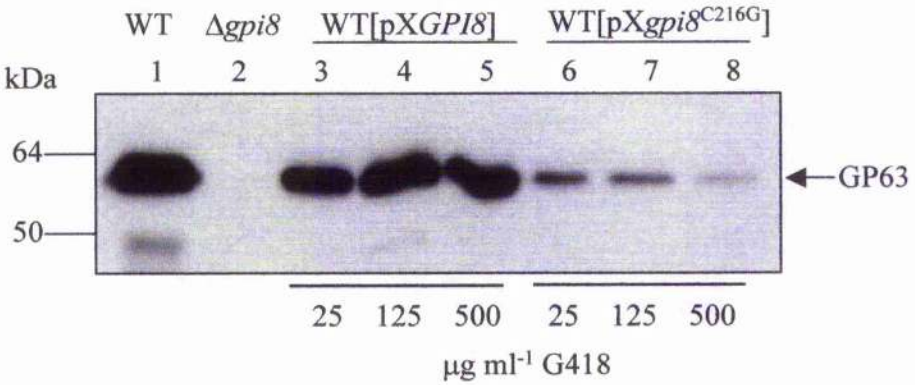
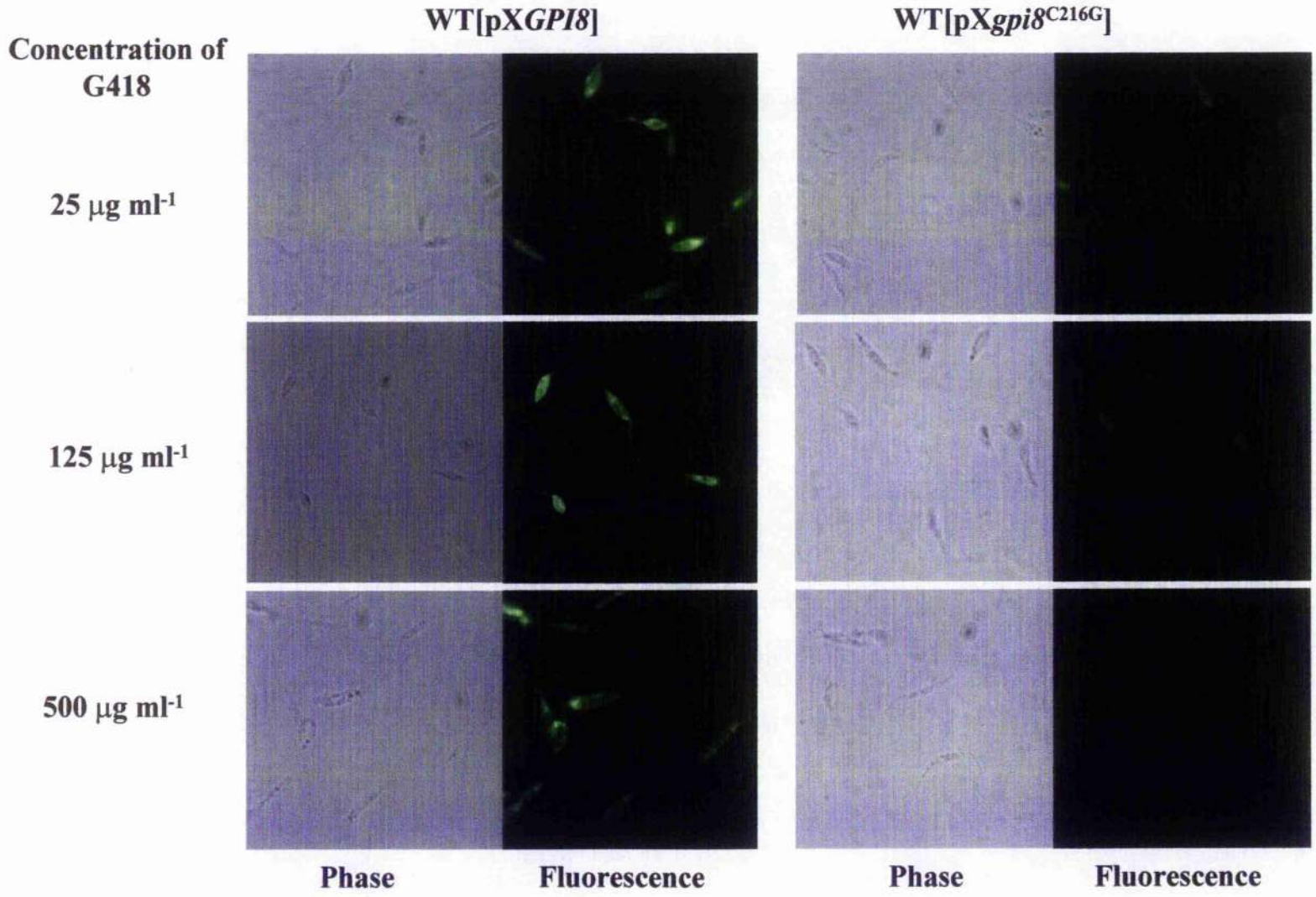


Figure 3.6: Analysis of GP63 expression in WT promastigotes expressing GPI8 and GPI8^{C216G} from episomes.

Western blot analysis of GP63 expression in *L. mexicana* lysates. Cell-lines WT[pX*GPI8*] (lanes 3-5) and WT[pX*gpi8*^{C216G}] (Lanes 6-8) were grown in increasing concentrations of G418 to select for increased plasmid copy number and a higher level of expression of GPI8 or GPI8^{C216G}. Lysates were prepared from 10^8 cells, and 10^7 cell equivalents per sample were electrophoresed by 12% SDS-PAGE, and electroblotted. Western blot analysis was performed with an α-GP63 monoclonal antibody used at a dilution of 1:50. GP63 is indicated.

Figure 3.7: Immunofluorescence detection of surface bound GP63 in wild type cells expressing either a functional or non functional form of GPI8 from an episome.

Cells expressing episomal copies of GPI8 were grown in increasing concentrations of G418 to increase episomal copy number, and hence GPI8 expression. *L. mexicana* promastigotes were fixed in 2.5% paraformaldehyde. Fixed cells were incubated with a GP63 primary antibody (L3.8) at a dilution of 1:100, and a FITC conjugated anti-mouse secondary antibody (Sigma) at a dilution of 1:500, to allow detection of surface bound GP63.


WT[pXGPI8]
WT[pXgpi8C216G]
Concentration of G418
25 μg ml-1
125 μg ml-1
500 μg ml-1
Phase
Fluorescence
Phase
Fluorescence

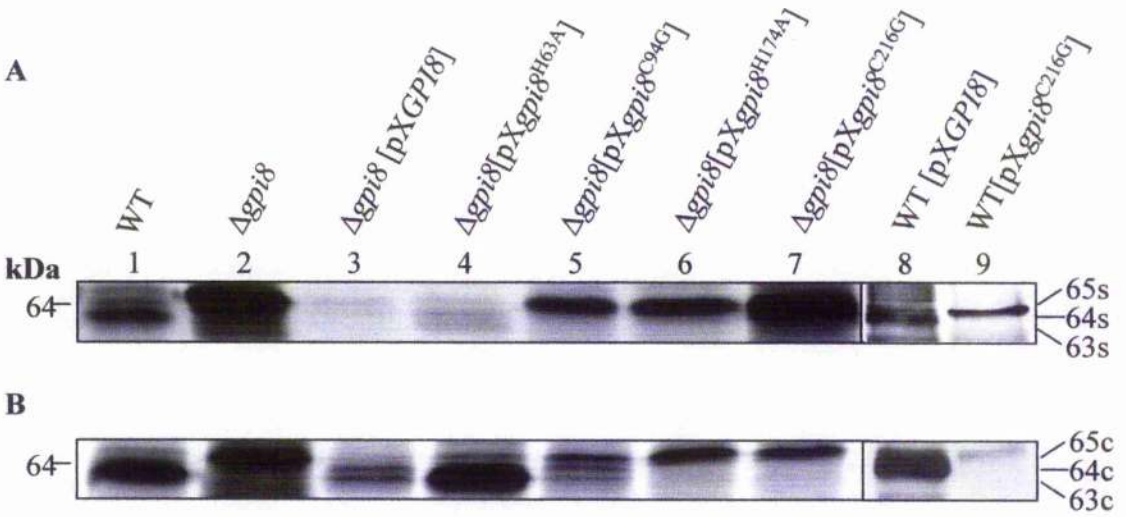


Figure 3.8: Secretion of GP63 from Δ*gpi8*. *Leishmania* promastigotes were cultured with ^{35}S-ExpreSS for 6 h. Samples from the medium (*Panel A*) or cell lysates (*Panel B*) were collected, immune-precipitated with α-GP63 antibody and electrophoresed on a 12% PAGE gel. Gels were scanned with a phosphor imager. Secreted GP63 (65s, 64s, 63s in kDa) and cellular GP63 (65c, 64c, 63c in kDa) are indicated.

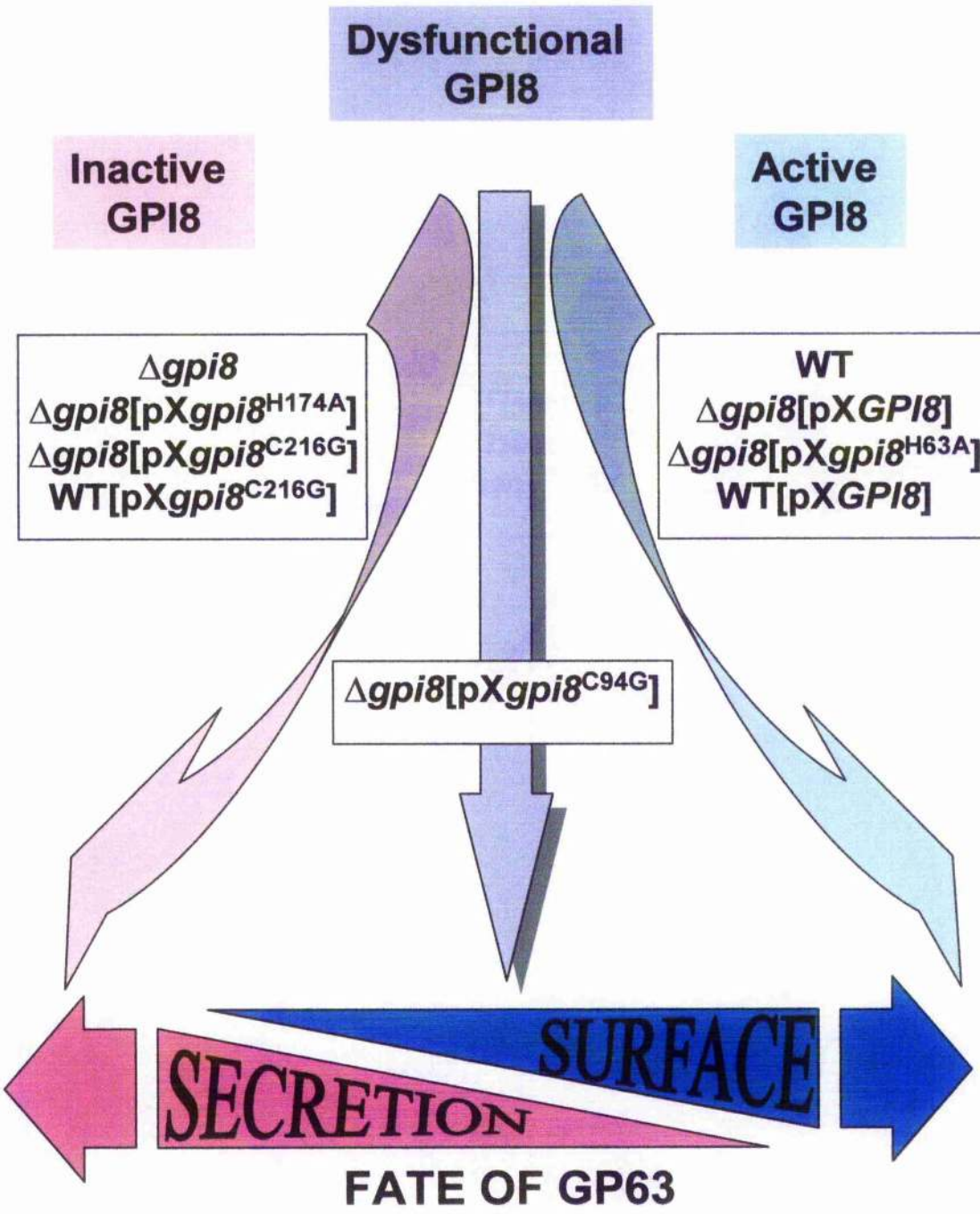


Figure 3.9: Summary of the activity of GPI8 based on the ultimate fate of GP63.

Cells with a fully functional GPI8 traffic GP63 to the cell surface, whilst cell lines without a functional GPI8 secrete large amounts of GP63 from the cell. The Δ*gpi8*[pX*gpi8*^{C94G}] cell line has a dysfunctional GPI8, and has an intermediate phenotype with secretion from the cell and transport to the surface.

Chapter 4

Characterisation of the trafficking and processing of the GPI-anchored protein GP63 in WT and Δ*gpi8* cell lines.

4.1 Introduction

GP63 is the major GPI-anchored protein of *Leishmania* promastigotes as described in detail in section 1.3. Previous studies of GP63 have identified functional domains characteristic of a GPI-anchored metalloproteinase by sequencing studies and site directed mutagenesis (Button and McMaster, 1988; McMaster *et al.*, 1994). These domains are thought to be processed during the trafficking of the protein to the cell surface (Figure 4.1), though the precise intracellular processing of GP63 in WT cells has not previously been fully analysed. It has been shown that modification of GP63 for secretion, by deletion of the C-terminal end, results in the release of an inactive protein using either a baculovirus expression system, or Cos-7 cells (Button *et al.*, 1993; McMaster *et al.*, 1994; Macdonald *et al.*, 1995). Mutation of the anchor addition site of *L. major* GP63, and expression in a *L. amazonensis* GP63 deficient cell line resulted in the secretion of an active protein (McGwire and Chang, 1996). In Chapter 3 it was demonstrated that both the processing and trafficking of GP63 is affected by the loss of GPI8 activity from the cells. The aim of this section of work was to characterise these differences in greater detail.

4.2 Comparison of processing in WT versus Δ*gpi8* cells

4.2.1 Sequence analysis of *L. mexicana* GPI anchored GP63

In order to characterise the processing of GP63 in the *L. mexicana* WT and Δ*gpi8* cell lines the protein's functional domains were identified. Previous studies have focused on *L. major* and *L. amazonensis*, and the multiple genes encoding GP63 have been shown to be highly conserved within and between species (Button and McMaster, 1988). *L. mexicana* is atypical, in that amastigotes express a non GPI-anchored form of GP63 termed C1 in a stage regulated manner (Medina-Acosta *et al.*, 1993). The C1 GP63 has been sequenced and shown to vary from *L. major* GP63 in its C-terminal sequence, and increased number of N-glycosylation sites (9 potential sites were identified) (Medina-Acosta *et al.*, 1993). The characteristic homology of the GPI-anchored promastigote

form (C2) of *L. mexicana* GP63 was confirmed by sequencing a copy of C2 GP63. A plasmid pSK-4 (a gift from Dr D. Russell, Washington University, renamed pGL454) containing GP63 cDNA from *L. mexicana* cloned into the *Eco*RI site of pBluescript SK (Medina-Acosta *et al.*, 1993) was sequenced and the predicted protein compared with GP63 from *L. major* and *L. amazonensis* (Figure 4.2).

The *L. mexicana* GPI-anchored form of GP63 is predicted to have a precursor protein of size 602 amino acids, whilst the mature peptide is predicted to be of size 477 amino acids. The functional domains were predicted either by use of the 'big-PI predictor' (Eisenhaber *et al.*, 1998), or by homology to domains identified in *L. major* and *L. amazonensis* GP63. An ER signal sequence is predicted from residues 1-37 (Eisenhaber *et al.*, 1998), homologous to those found in *L. major* and *L. amazonensis* (Button and McMaster, 1988; Button *et al.*, 1993). A Pro-region is predicted from residues 38 to 100 based on similarity to *L. major* and *L. amazonensis* (Button and McMaster, 1988; Macdonald *et al.*, 1995). The GPI anchor addition site is predicted to be at residue 577, with a hydrophobic region from 586-602 (Eisenhaber *et al.*, 1998). 3 potential N-glycosylation sites are predicted at positions 300, 407 and 534, these sites are conserved in both *L. major* and *L. amazonensis*. All 3 sites have been shown by site-directed mutagenesis in *L. major* to be N-glycosylated (McGwire and Chang, 1996). The zinc-binding domain is also conserved at position 262 to 266 based on homology to the *L. major* and *L. amazonensis* sequences (McMaster *et al.*, 1994). The identity between *L. mexicana*, *L. major* and *L. amazonensis* GPI-anchored GP63 is 76.9%, and the similarity between *L. mexicana* and *L. major* 82%, and between *L. mexicana* and *L. amazonensis* is 90%. This demonstrates that, unlike the amastigote form of GP63, the *L. mexicana* promastigote form of GP63 is highly conserved between species.

4.2.2 Pulse-chase labelling of cells.

Lack of an active GPI8 effects the processing and trafficking of GP63 when compared with WT cells. To examine these variations in more detail WT promastigotes, Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] cells were analysed by pulse-chase labelling. Cells were metabolically labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 300 minutes. GP63 was immune-precipitated from the cells or culture medium and analysed by SDS-PAGE. The processing of GP63 within the

cells was also examined by partitioning the cells into soluble and membrane associated fractions by either extraction with TX-114 or $NaCO_3$ (Figure 4.3).

The data shown in figure 4.3 is representative of a number of repeated experiments. Pulse-chase labelling of cells and extraction with TX-114 (Figure 4.3, A) demonstrated that in WT cells GP63 partitioned exclusively into the membrane associated fraction at all time points. At time 0 minutes of chase, the major isoform of GP63 was 65c (cellular). After 20 minutes, the majority of 65c had been chased into 64c and by 40 minutes 64c was in the process of being chased into 63c. By 180 minutes all detectable label was in the 63c isoform. In contrast to wild type cells, only the 65c isoform was detected in the Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] lines (Figure 4.3, A). Moreover, most of this 65c form remained in the soluble phase in Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] cells. $NaCO_3$ extraction of pulse-chase labelled cells produced similar results (Figure 4.3, B). In WT cells GP63 partitioned exclusively into the membrane associated fraction at each time point. During the chase period the protein underwent a size change from a 65c isoform present at time 0, to reach a 63c isoform at time 180 minutes. In the Δ*gpi8* cells only the 65c isoform of GP63 was detected throughout the chase period. This 65c isoform partitioned into both the soluble and membrane fractions, differing from the result seen following TX-114 fractionation.

2 forms of GP63 were secreted from WT cells, a 65s (secreted) and 63s form (Figure 4.3, C). Only a 65s form of GP63 was secreted from the Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] cell lines. A low level of ^{35}S-labelled GP63 was secreted from wild type parasites, while high levels of secreted GP63 could be detected over a 180 minute chase period for both Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] (Fig 4.3, C). In order to quantify this difference the level of GP63 secretion was measured using a Typhoon Phosphor-imager, and quantified using the Image Quant programme (Molecular Dynamics). To allow direct comparison between WT and Δ*gpi8* cells the results from individual experiments were used in which both cell lines were labelled in tandem and all conditions such as cell numbers, quantity, activity of ^{35}S and exposure times to the phosphor-imager were identical. The results from a 300-minute pulse-chase experiment are shown (Figure 4.4) and are representative of a number of individual experiments. After 300 minutes the level of GP63 secretion from WT cells was estimated to be only 13% of that secreted

from Δ*gpi8* cells, with the $t_{1/2}$ for Δ*gpi8* GP63 secretion estimated to be 120 minutes (Figure 4.4). This clearly demonstrates the higher rate of secretion of newly synthesised GP63 in cells with a non-functional GPI8.

Repetition of pulse-chase experiments and Triton X114 fractionation (Figure 4.3A) consistently produced the same results, and suggested that the intracellular distributions of GP63 within WT and Δ*gpi8* cells differed. A plot of the distribution of GP63 within the two cell lines confirmed this (Figure 4.5). The two graphs were calculated from data from a single pulse-chase experiment and were therefore directly comparable. The graphs show some fluctuations due to experimental error, particularly with regards to the WT membrane fractions, however the general trends can be interpreted and are representative of a number of experiments. In the WT cells GP63 remained almost exclusively in the membrane fractions, the small quantities of GP63 secreted from the cell were comparable to the amount of material found in the soluble cell fraction. In the Δ*gpi8* cell line intracellular GP63 remained in the soluble fraction, and this amount decreased during the chase period as GP63 was secreted from the cell. A small amount of GP63 remained in the membrane-associated fraction. The graphs clearly demonstrate the difference between the intracellular distribution of GP63 in WT and Δ*gpi8* cells.

To investigate the possibility that the expression levels of GP63 differed between the 2 cell lines the level of total expression was calculated by combining the quantity of GP63 present in each cell fraction (Figure 4.6). The pattern of GP63 protein expression was similar, though expression from the Δ*gpi8* cells was approximately 20% lower than in WT cells (approximately 8,000,000 units compared to 10,000,000 at time 300 minutes). It is possible that this is due to some degradation of the protein intracellularly.

4.3 Processing events within WT and Δ*gpi8* cell lines

The results from the pulse-chase labelling experiments clearly demonstrate that GP63 in Δ*gpi8* cells is processed differently than GP63 in WT cells. The 65c form was speculated to represent a precursor form of GP63, which underwent further processing in WT cells to reach the mature 63c form. In the Δ*gpi8* cells it appeared that the 65c form was not processed to the fully mature form. Thus lack of GPI-anchor addition to the protein appeared to prevent further downstream processing. To investigate this

hypothesis the processing of GP63 in both WT and the Δ*gpi8* cells was examined in greater detail. The removal of the C-terminal GPI-signal sequence, addition of the GPI anchor, variations in glycosylation, and activation by removal of the pro-region were all examined.

4.3.1 Timing of anchor addition

It was speculated that the 65c form of GP63 present in both WT and Δ*gpi8* cells represented an unanchored form of GP63. The timing of GPI anchor addition during the processing of GP63 was examined by determining which forms of GP63 had a GPI anchor. Treatment of a GPI-anchored protein with the enzyme phosphatidylinositol phospholipase C (PI-PLC), cleaves the GPI-anchor at the PI moiety, and subsequent TX-114 fractionation results in the protein partitioning into the soluble, rather than the membrane phase. WT promastigotes were labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 180 minutes. Samples were taken at time 0 minutes, 40 minutes and 180 minutes and lysates treated with or without PI-PLC followed by TX-114 fractionation and GP63 immune-precipitation (Figure 4.7). At time 0 minutes of chase only the major 65c protein was present. This isoform partitioned into the membrane-associated fraction (lane 2), but after PI-PLC treatment was found in the soluble fraction (lane 3) consistent with the GPI anchor having been removed. This pattern of results was repeated with samples taken at time 40 minutes, and time 180 minutes, all protein isoforms detected were present in the membrane fraction, but subsequent to PI-PLC treatment were detected in the soluble fraction. This demonstrated that in WT cells all isoforms of GP63 detected by pulse-chase labelling were GPI-anchored, and that GPI anchor addition occurred very rapidly after translocation into the ER. It should also be noted that a fourth minor isoform of GP63 could be detected (lanes 6 and 7). As all the detected isoforms of GP63 were GPI-anchored, additional post-translational modifications, such as glycosylation and activation must occur to process the 65c isoform to the 63c isoform.

4.3.2 Glycosylation

ConA precipitation

Glycosylation of GP63 was examined by testing if the protein bound to the lectin Concanavalin A (ConA), which interacts with the tri-mannose core common to all N-

linked glycans. Cells were grown to late log phase, the medium collected and proteins ConA precipitated using ConA sepharose beads. In a separate experiment $1x10^7$ cells were collected and proteins ConA precipitated from the cell lysate. Samples were electrophoresed on 12% SDS-PAGE gels, electroblotted and GP63 was detected by western blotting with a GP63 antibody (Figure 4.8). A control experiment using sepharose 4B beads in place of ConA sepharose beads demonstrated that GP63 did not bind to Sepharose beads non-specifically (data not shown). Proteins were detected in the ConA precipitated medium and cell samples from both cell lines (Figure 4.8). A much higher level of glycosylated GP63 bound to ConA from WT cell lysate material (lane 5) than from Δ*gpi8* cell lysates (lane 6). In contrast glycosylated GP63 was present at a lower level in the medium of WT cells (lane 3) compared with that from Δ*gpi8* cells (lane 4), and the size of GP63 precipitated was larger in the Δ*gpi8* samples. This demonstrates that GP63 is N-glycosylated in both WT and Δ*gpi8* cells and that N-linked glycosylation can occur in the absence of GPIT activity.

Tunicamycin Treatment

To examine the effect that loss of N-glycosylation would have on GP63 processing, the two cell lines were grown in medium containing 5 μg ml^{-1} tunicamycin to inhibit N-linked glycan formation. Pulse-chase labelling was used to examine the processing of GP63 under these conditions (Figure 4.9, A and B). GP63 was processed differently in WT cells grown in the presence or absence of tunicamycin. A smaller form of GP63 (approximately 63 kDa, designated 63ct - cellular material with tunicamycin) was present at time 0, and this was chased into a 60 kDa (60ct) after 180 minutes. Only a single minor intermediate form of GP63 was identified in cells grown in the presence of tunicamycin, compared to the one major (64c) and one minor protein identified in normally grown WT cells. The Δ*gpi8* cells grown in the presence of tunicamycin also expressed a smaller GP63 (63ct). However, this was not chased to a 60 kDa form. The size difference between the proteins isolated at time 0 from cells grown in the presence and absence of tunicamycin correlates with the lack of N-glycosylation. The lower number of detectable intermediate forms present in WT cells grown in the presence of tunicamycin, indicates that one of the isoforms detected during GP63 maturation may be a result of N-glycan processing. GP63 could not be detected in the medium of wild type cells grown in the presence of tunicamycin (Figure 4.9, B). The level of GP63

detected in the medium of the Δ*gpi8* cell line grown in the presence of tunicamycin was barely detectable over background levels, contrasting with the high levels of GP63 secreted by the Δ*gpi8* cells grown under normal conditions. This suggests that N-linked glycosylation is important for the secretion of GP63, from both WT and Δ*gpi8*.

PNGaseF treatment

The timing of glycosylation was further examined by PNGaseF treatment of GP63. PNGaseF cleaves N-glycans between the innermost GlcNAc and asparagine residues. WT and Δ*gpi8* cells were labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 180 minutes. Samples were taken at appropriate time points and cell lysates TX-114 fractionated and GP63 immune-precipitated. The membrane fraction from the WT cells, and the soluble fraction from the Δ*gpi8* cells were taken and each sample was divided in two, one half was treated with PNGaseF, and the other half mock treated. Samples were then electrophoresed by 12% SDS-PAGE, and scanned with the phosphor-imager (Figure 4.10). At each time point in both cell lines all GP63 isoforms decreased in size subsequent to PNGaseF treatment. This size decrease correlates with the loss of N-glycosylation and confirms the results from the tunicamycin experiment that all forms of GP63 identified by pulse-chase labelling are N-glycosylated.

Four isoforms of GP63 were detected in WT cells subsequent to PNGaseF treatment (63kDa, 62kDa, 61kDa and 60kDa, designated cp-cellular material with PNGaseF). In Δ*gpi8* the single 65c form of GP63 present at all time points resolved into 2 smaller forms (63cp and 62cp) subsequent to PNGaseF treatment. This was similar to the situation in WT cells at time 0 minutes of chase. This suggests that the 65c form of GP63 may be composed of 2 different isoforms of GP63. In WT cells these 2 isoforms appear to be processed simultaneously, to reach a 63c form, whilst in Δ*gpi8* cells both forms remain unprocessed.

4.3.3 Activation of GP63 by removal of the Pro-region.

GP63 is activated as a metalloproteinase by the removal of the pro-region (McMaster *et al.*, 1994; Macdonald *et al.*, 1995). GP63 is known to be active when it reaches the surface of the cell, however the precise mechanism and timing of this activation is not

known (Macdonald *et al.*, 1995; McGwire and Chang, 1996). In order to establish if the secreted form of GP63 from Δ*gpi8* cells was active, the activity of GP63 toward gelatin was examined by substrate SDS-PAGE. Previous studies have demonstrated that GP63 activity can be detected in WT cell lysate material by this method (Funk *et al.*, 1994; McGwire and Chang, 1996).

In a preliminary experiment cells were grown to late log phase, washed and pelleted. The cells were prepared as for normal SDS-PAGE, with the exception that the samples were not boiled prior to loading. Samples were electrophoresed on a 10% acrylamide, 0.1% gelatin gel, and subsequently the gel divided in two, and one part electro-blotted to allow detection of GP63 by western blotting. The other section of the gel was washed in 2.5% TX-100 to remove excess SDS, and incubated overnight in 0.1M Na Acetate pH 5.5, 1mM DTT at 37°C. The gel was stained in Coomassie blue R280 to allow visualisation of the gelatinolytic activity (Figure 4.11). Activity was detected in both Δ*gpi8* (lanes 1 and 2), and WT cell lysates (lanes 3-5). Differing cell equivalents ($5x10^6$, $2x10^6$, or $1x10^6$) were loaded to determine the sensitivity of the assay, and the same banding pattern was present for each (compare lanes 3, 4 and 5). The western blot of the remaining portion of the gelatin gel demonstrated that, while electro-transfer of proteins from gelatin gels was possible, the transfer efficiency was greatly reduced compared to normal SDS-PAGE gels. Both the incomplete transfer of the pre-stained higher molecular weight markers and Ponceau S staining of the blotted membrane, indicated that transfer of larger proteins was very inefficient. GP63 was detected in WT cells when $5x10^6$ cell equivalents were loaded (Figure 4.11, lane 6). Stained markers run on the gel were not considered an accurate indicator of size as samples were not denatured prior to loading, however non-denatured GP63 had a mobility close to that of the 64kDa marker. The markers were used to accurately align the activity gel with the western blot, allowing the identification of GP63 on the gelatin gel (Indicated in figure 4.11). Active GP63 is present only in the WT cell lysates. The identity of the larger protein with gelatinolytic activity present in both WT and Δ*gpi8* cell lysates is not known. A search of the MEROPS database (Rawlings *et al.*, 2002) for other metalloproteases present in *Leishmania* suggest that in *L. major* (the *Leishmania* species undergoing sequencing by the genome project) 3 ORFs have been identified which might encode for putative metalloproteinases.

Several experiments were performed to test if secreted GP63 was active. Firstly Vivaspin 20ml concentrators (Nalgene) were used to concentrate medium samples. However when the concentrated samples were electrophoresed on gelatin gels a high level of background protein was present, caused by the concentration of proteins from FCS, which was also present in the medium. The level of background proteins masked the detection of gelatinolytic activity. Secondly secreted GP63 was purified from the medium by immune-precipitation with a GP63 antibody. This method also caused masking of activity due to the high level of background proteins visable on the gel. This was due to cross-contamination with both the antibody and non-specific proteins from the FCS which co-precipitated. This method had the added disadvantage in that the presence and level of GP63 in the samples could not be determined by western blotting, as both the L3.8 GP63 antibody used for immune-precipitation and the antibody used for GP63 detection by western blotting, were mouse monoclonals, and so cross-reacted. Thirdly GP63 was purified from the medium by ConA precipitation. This method had an advantage in that ConA sepharose was available in sufficient quantities to allow purification from a large volume of medium. Secreted GP63 was readily detected in both WT and Δ*gpi8* samples, by western blotting of medium samples purified by this method (Figure 4.12, A). When the same samples were run on a gelatin gel, assessment of the gelatinolytic activity of the ConA purified medium samples was not possible (Figure 4.12, B). Glycosylated proteins present in the serum had co-affinity purified with the secreted GP63, and the high level of background protein prevented the detection of gelatinolytic activity.

Two gelatin gels were compared to assess GP63 activity in cell lysates under different activation conditions, as a pH of 8.0 was considered closer to the pH optimum at which GP63 was active (Ip *et al.*, 1990). One gel was incubated with 0.1M Na Acetate pH 5.5, 1mM DTT, the other with 50mM Tris-HCl pH 8.0 at 37°C overnight. The banding pattern on the gels was similar but a greater level of activity was present on the gel incubated at pH 8.0 (Data not shown). This condition was therefore used in all subsequent activity experiments to increase the sensitivity of the assay.

To prevent the contamination of medium samples with proteins from FCS, cells were grown to mid-log phase in normal conditions, washed in PBS, resuspended in HOMEM

in the absence of FCS, and allowed to grow for a further 6 hours. Medium samples were then collected and analysed. Figure 4.13 shows a representative experiment. In this case $1.7x10^8$ WT and $3.4x10^8$ Δ*gpi8* cells were grown to mid log phase, washed and grown in HOMEM in the absence of FCS for 6 hours. Both cell and medium samples were ConA precipitated, examined for activity by electrophoresis on gelatin gels, and western blotted to detect GP63. All samples had gelatinolytic activity (Figure 4.13, A). The characteristic protein thought to be GP63, as assessed by motility, was present in both the WT cell and medium samples (Lanes 1 and 3). No similar sized protein was present in either the Δ*gpi8* cell or medium samples (Lanes 2 and 4). Some activity was detected in the Δ*gpi8* medium sample, however this protein had a faster electrophoretic mobility than the protein predicted to be active GP63 in WT cells. This active protein may be an isoform of GP63.

Western blotting with an αGP63 antibody detected GP63 in all 4 samples (Figure 4.13, B). The GP63 isoform secreted from Δ*gpi8* cells had a slower electrophoretic mobility than the WT isoform when electrophoresed by SDS-PAGE (Compare lanes 7 and 8). This result was repeated when the samples were not denatured prior to electrophoresis. The faster mobility of the gelatinolytically active protein detected in the medium sample from the Δ*gpi8* cells (Figure 4.13, A, lane 4), and the failure to detect a similar sized protein by western blotting suggested that it was not an isoform of GP63. However, a western blot of these samples electrophoresed on a gelatin gel was only able to detect GP63 in the WT cell samples due to the inefficiency of transfer. It was therefore not possible to establish conclusively the mobility of the GP63 isoform secreted from Δ*gpi8* cells, when electrophoresed under these conditions.

Western blotting and detection of GP63 demonstrated that GP63 was present at reduced levels in the Δ*gpi8* cells and medium compared to WT, when grown in medium lacking FCS (Figure 4.13, B). The level of GP63 secretion from the Δ*gpi8* cell line was higher than that from WT cells when grown under normal conditions (Figure 4.4). It is possible that Δ*gpi8* cells are unable to grow in conditions lacking FCS. To test this WT and Δ*gpi8* cells were grown in medium with or without FCS for 6 hours, and the number of dead cells counted. There were a higher number of dead cells in the medium lacking FCS, and in this medium Δ*gpi8* cells showed a greater level of cell death

compared to the WT cells. A growth curve of cells grown in these conditions would provide more accurate evidence for this. Repeated experiments in which the conditions of cell growth, such as cell density, FCS concentration, and culture volume, were altered each failed to conclusively detect active GP63 secreted from the Δ*gpi8* cell line. It was not clear if this failure was due to the low levels of GP63 secreted when cells were grown in the conditions optimal for the assay method, or if GP63 was secreted in an inactive form.

It was possible that the failure to conclusively demonstrate GP63 activity in the secreted protein from Δ*gpi8* cells was because the protein was inactive. Metalloproteinases can be activated by treatment with $HgCl_2$, as this compound disrupts the cysteine-zinc complex which exists between the pro-region and zinc-binding domain (Springman *et al.*, 1990). This method has previously been used to activate GP63 secreted using a baculovirus expression system (McMaster *et al.*, 1994; Macdonald *et al.*, 1995). Cells were grown to late log phase, washed and incubated in 5mls of HOMEM lacking FCS for 4 hours and the medium collected and pre-cleared prior to ConA precipitation. The eluted samples were treated with or without 2μM $HgCl_2$ at 37°C for 1 hour prior to electrophoresis on a 10% SDS-PAGE with 0.1% gelatin, and one gel was treated for gelatinolytic activity (Figure 4.14, A). An active enzyme was detected in the Δ*gpi8* medium sample subsequent to $HgCl_2$ treatment. This gelatinolytically active protein was the same size as that detected in the WT sample, and was barely detectable in the untreated sample (compare lanes 4 and 5). Treatment of the WT sample with $HgCl_2$ appeared to inactivate GP63, however subsequent experiments did not support this finding. WT cell lysates were treated with increasing concentrations of $HgCl_2$ but active GP63 was still detected as determined by gelatinolytic activity. The apparent loss of activity seen in this experiment was therefore concluded to be due to experimental error. A second gelatin gel was western blotted using a GP63 antibody, but GP63 was not detected in either the WT or Δ*gpi8* samples (data not shown). The purified medium from an estimated 1.5 $x10^7$ cells was loaded, and the level of GP63 present was thought to be below detectable levels.

$HgCl_2$ treatment of the inactive protein prior to electrophoresis would result in the activation and the possible removal of the pro-region by proteolysis. The activated

Δ*gpi8* protein would be smaller in size, and therefore be predicted to have a faster electrophoretic mobility than the inactive enzyme. However, a second gelatinolytically active enzyme was present in the Δ*gpi8* untreated sample (Figure 4.14 A, lane 4), with increased mobility compared to the activated enzyme (Lane 5). This protein was absent following $HgCl_2$ treatment, and it was possible that this was an isoform of GP63 which could be fully activated by $HgCl_2$ treatment, which caused it to run with decreased mobility under denaturing conditions. Activation of Δ*gpi8* GP63 subsequent to electrophoresis on a gelatin gel would be expected to identify a different sized isoform of GP63 from WT. This would provide further evidence that an inactive protein was being activated by pro-region removal.

Cells were grown in the absence of FCS for 4 hours and the medium pre-cleared and ConA precipitated. WT cell lysate ($5x10^6$ cell equivalents) and medium samples from an estimated $2x10^8$ cells were electrophoresed on 10% SDS PAGE gels containing 0.1% gelatin. The gels were treated as previously, with the exception that one gel was also incubated with 20mM of the metalloproteinase inhibitor 1-10 phenanthroline, and a second gel incubated with 4μM $HgCl_2$ during washing and activation steps (Figure 4.14, B). Gelatinolytic activity was present in all samples. Incubation with 1-10 phenanthroline reduced activity in the medium, and cell lysate samples (Compare lanes 1 and 4, 2 and 5 and 3 and 6). Incubation with $HgCl_2$ appeared to increase activity in the Δ*gpi8* medium sample (Compare lanes 1 and 7). This increase in activity appeared to be greatest in a protein that had slower electrophoretic mobility than the protein predicted to be GP63 in the WT cells. It was not clear if all the active proteins visible in the Δ*gpi8* sample were different isoforms or degradation products of GP63. It is possible that Δ*gpi8* cells secrete a second glycosylated protein with gelatinolytic activity.

The results from these experiments, suggested that the secreted form of GP63 from WT cells was active whilst the protein secreted from Δ*gpi8* cells was inactive. Treatment with $HgCl_2$ appeared to increase activity in the Δ*gpi8* samples. However the technical difficulties associated with purifying large quantities of secreted protein from medium containing FCS, and the poor growth of the Δ*gpi8* cells in medium lacking FCS, meant that it was not possible to conclusively identify active or inactive GP63 secreted from

Δ*gpi8* on gelatin gels. The results also showed a large degree of variability, this poor reproducibility may have been a due to the low levels of GP63 activity being outwith the limits of detection for the assay. Previous work has studied the gelatinolytic or caseinolytic activity of GP63 in cell lysate material by gel electrophoresis (Funk *et al.*, 1994; McGwire and Chang, 1996). Where secreted GP63 was examined GP63 was secreted from either Cos7 cells or insect cells in a baculovirus expression system, into serum free medium and the protein purified by affinity purification (Button *et al.*, 1993; Macdonald *et al.*, 1995). The activity of GP63 has also been studied using succinylated casein as a substrate, and measuring GP63 activity by absorbance (Macdonald *et al.*, 1995; McGwire *et al.*, 2002). In order to further investigate the presence or absence of GP63 activity in secreted forms of GP63, the QuantiCleave Protease Assay Kit (Pierce) was used. Preliminary experiments were unsuccessful, and time constraints prevented the production of conclusive data. In theory this method would provide a more sensitive method of detecting metalloproteinase activity, and would remove the problem of FCS causing excessive background interference.

4.4 Trafficking of GP63 from the cell

The results suggest some differences in the both the processing and trafficking of GP63 within both the WT and the Δ*gpi8* cell lines. Whilst GPI-anchor addition was clearly the most obvious difference in the processing of GP63 in the 2 cell lines, an unanchored isoform was not one of the processing intermediates detected in WT cells in this study. PI-PLC treatment demonstrated that all WT isoforms detected were GPI-anchored, and anchor addition occurred very rapidly in WT cells. It appeared that lack of a GPI anchor was affecting the downstream processing of GP63. It seems possible that this variation in processing is a result of GP63 trafficking through different pathways, or different compartments in the same pathway within the two cell lines. It is also possible that two different pathways for GP63 trafficking are present within the WT cell line.

The trafficking of GP63 from the cell was further examined, initially by examining the secretion of GP63 from WT cells. The results from pulse-chase labelling experiments clearly indicated that 2 forms of GP63 were secreted from WT cells (See figure 4.3, C, time point 180 minutes). The 65s isoform was visible within 20 minutes in WT, Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] cells, whilst the 63s protein was detected only after 80

minutes. This suggests that the kinetics of secretion for the 2 isoforms are different and indicate that the two isoforms may be trafficked through 2 different pathways. The 65s form of GP63 may be a non-GPI-anchored isoform that was secreted directly from the cell. The 63s form may be a mature processed form of GP63, released from the cell surface by cleavage of the GPI-anchor.

4.4.1 TX-114 fractionation

In an attempt to examine the presence or absence of a GPI anchor on the secreted forms of GP63 from WT cells, medium samples were subjected to TX-114 fractionation and PI-PLC treatment. WT and Δ*gpi8* cells were grown to mid log phase and 1x 10^8 cells were labelled with ^{35}S-ExpreSS for 6 hours, the medium collected, and pre-cleared to remove cellular debris. Samples were divided into 2 and one half treated with PI-PLC prior to TX-114 fractionation and immune-precipitation with a GP63 antibody (Figure 4.15). The low level of GP63 secretion in WT cells meant that the quantity of protein obtained was close to the limits of detection. However it was clear that the 2 isoforms of GP63 partitioned into different phases. The 65s isoform was detected in the soluble phase (Lane 1), whilst the 63s isoform partitioned into the membrane associated fraction (Lane 2). After PI-PLC treatment the 63s form of GP63 was absent from the membrane fraction and appeared in the soluble fraction indicative of the presence of a GPI anchor. This result suggests that the 65s form is secreted as a soluble form in the WT cell line, whilst the 63s form is secreted or released into the medium with a GPI anchor. This was an unexpected result as it was expected that the 63s form present in WT medium was released from the cell surface by cleavage of the GPI anchor, and thus would partition into the soluble phase of TX-114. It seems possible that the 63s form of GP63 was contamination from cellular material, though medium samples were pre-cleared prior to immune-precipitation to prevent this. If cellular contamination were responsible for the presence of the 63s protein present in the medium, then the relative amounts of 63s versus 65s would be expected to be higher to reflect the high levels of the 63c isoform of GP63 present within the cell. Cellular contamination would also be expected to release other GPI-anchored GP63 intermediates, such as the 64c isoform, into the medium, but this protein was not detected. This suggests that the GPI-anchored form of GP63 was not present in the medium due to cellular contamination. In WT cells

a 65kDa unanchored isoform of GP63 is secreted into the medium within 20 minutes, and a 63kDa GPI-anchored isoform is released into the medium within 80 minutes.

4.4.2 Brefeldin A treatment

To examine the intracellular trafficking of GP63 in more detail, cells were grown in Brefeldin A (BFA). BrefeldinA inhibits secretion by disruption of the Golgi body (Donaldson *et al.*, 1992). It was thought that comparison of the trafficking of GP63 in WT and Δ*gpi8* cells grown in BFA would allow some elucidation of whether GP63 was trafficked through separate pathways, and whether processing events occurred in pre or post-Golgi compartments.

Cells were grown with or without 10μg ml^{-1} BFA for 4 hours prior to pulse-chase labelling with ^{35}S-Express, and immune-precipitation of GP63 as previously described. BFA was also added to the medium during the chase period as appropriate. A comparison between treated and untreated cells (Figure 4.16 panel A), shows there is no apparent difference in the processing of GP63, as assessed by the number of intermediates formed, or the timing of this process. In both treated and untreated WT cells the 65c isoform was processed through intermediate stages to reach the 63c isoform after 180 minutes of chase as seen previously (see figure 4.3). In both treated and untreated Δ*gpi8* cells the 65c isoform of GP63 remained through out the chase period with no further processing. The secretion of GP63 from both the WT and Δ*gpi8* cell lines was apparently unaffected by the inhibitor BFA (Figure 4.16, B). WT cells secreted a low level of the 65s and 63s isoforms, whilst the Δ*gpi8* cell line secreted a high level of the 65s isoform

The experiment was repeated with WT cells grown in 50μg ml^{-1} BFA (Data not shown). Growth of cells in an increased concentration of the inhibitor still had no discernible effect on the processing of GP63. It is possible that GP63 processing occurs pre-Golgi and that BFA treatment would not influence the processing of GP63, but would have an effect on the trafficking of the protein to the cell surface. To test this possibility, WT cells were grown in BFA and subsequently treated with PI-PLC to check if labelled protein could be cleaved from the cell surface. Whilst PI-PLC treatment did not work efficiently, a small amount of GP63 could be detected from both

treated and untreated cells. It was concluded that BFA does not influence the intracellular processing, trafficking or secretion of GP63 in *L. mexicana.*

4.5 Discussion

Previous studies have shown that GP63 is GPI-anchored (Schneider *et al.*, 1990) and N-glycosylated (Button *et al.*, 1989; McGwire and Chang, 1996). The protein is activated intracellularly by the removal of the pro-region (Macdonald *et al.*, 1995; McGwire and Chang, 1996) and trafficked to the cell surface via the flagellar pocket (Overath *et al.*, 1997; Weise *et al.*, 2000). This study has examined in detail the intracellular processing of GP63 in WT cells, and compared this with the situation in cells with a defect in GPI-anchor addition. Pulse-chase labelling experiments demonstrated that in WT cells GP63 undergoes a series of intracellular processing steps to reach its mature 63kDa GPI-anchored form (63c). One major and one minor intermediate form were identified during the intracellular processing from the 65c to the 63c form. In contrast within the Δ*gpi8* and Δ*gpi8*[pX*gpi8*^{C216G}] cell lines the 65c GP63 isoform undergoes no detectable form of modification, as assessed by size change, instead the 65kDa precursor is secreted from the cell. Loss of GPI8 activity, and the associated loss of GPI anchor addition therefore effects the intracellular processing of GP63.

TX-114 and $NaCO_3$ fractionation demonstrated that GP63 had a different intracellular distribution within WT cells lines when compared with cells lacking a functional GPI8. Within WT cells GP63 partitioned exclusively into the membrane associated phase. Within the Δ*gpi8* cell line GP63 partitions into the soluble phase as determined by TX-114 fractionation, whilst subsequent to $NaCO_3$ extraction the majority of the protein partitions into the soluble phase though some remains within the membrane associated fraction. The difference in the intracellular distribution of GP63 in the Δ*gpi8* cells subsequent to the different extraction methods may reflect the stringency of TX-114 compared to $NaCO_3$ extraction. TX-114 partitions GPI-anchored proteins and embedded membrane proteins, from soluble proteins whilst the NaCO3 method is less stringent and may also partition membrane-associated proteins into the membrane fraction. This may include GP63 associated with the membrane via the small C-terminal hydrophobic domain, suggesting a subset of the GP63 population remains

membrane-associated in the Δ*gpi8* cells. Both TX-114 and $NaCO_3$ fractionation demonstrate a large proportion of GP63 remains within the soluble fraction of the Δ*gpi8* cells, differing from the situation in WT cells whereby GP63 remains exclusively in the membrane fraction. Differences in the intracellular distribution of GP63 between the WT and Δ*gpi8* cell lines, and the secretion of high levels of the protein into the medium of the Δ*gpi8* cells indicate that lack of GPI8 activity affects the trafficking of GP63. It therefore appears that prevention of GPI-anchor addition effects both the further processing and trafficking of GP63. This implies that the GPI-anchor has an important role on the forward transport of the protein.

Prior to this study the precise details of the processing of GP63 in WT cells had not been examined. However the finding that in WT *L. mexicana* the major GPI-anchored protein is processed from a 65kDa GPI-anchored precursor, through intermediate forms to reach a 63kDa mature form is also supported by similar recently published work.

A study of *L. mexicana* GP63 has identified 3 GP63 isoforms (P1, P2 and P3) by pulse-chase labelling and TX-100 fractionation (Ralton *et al.*, 2002). P1 and P2 were recovered in the first 30 minutes of the chase period, P3 was detected after the first 30 minutes of chase. Biotin labelling demonstrated that P1 remained in the internal membranes, whilst P3 was found predominantly on the surface, though a small proportion remained internally. The P1 isoform is thought to be a GPI-anchored pro-form, whilst P3 is described as the major GPI-anchored isoform of GP63 (Ralton *et al.*, 2002). Work on *L. chagasi* identified 2 processing forms of GP63 of 66kDa, and 63kDa (Yao *et al.*, 2002). These were initially speculated to be isoforms of GP63 from two separate genes. An *L. donovani* attenuated cell line was used, which expressed only a low amount of GP63. Pulse-chase labelling of this *L. donovani* GP63 deficient cell line transfected with a single copy of *L. chagasi* GP63, and immune-precipitation with a *L. chagasi* GP63 specific antibody demonstrated a 66kDa pre-cursor was processed to a mature 63kDa form. This result was repeated when genes for any of the 3 different forms of *L. chagasi* GP63 (*MSPS1*, *MSPS2*, and *MSPL*) were expressed in the cell line. PI-PLC and N-glycanase treatment demonstrated that both forms of GP63 were GPI-anchored and N-glycosylated. It was speculated that the processing event was due to the removal of the pro-region (Yao *et al.*, 2002).

Processing of GP63 in a GPI biosynthesis mutant has also recently been examined. An *L. mexicana* cell line, DIG1, with a defect in the addition of α1-6 linked mannose to the glycan backbone common to both GIPLs and GPI anchors has recently been isolated (Naderer and McConville, 2002). The processing of GP63 was analysed in this cell line and it was shown that an isoform described as a non-GPI-anchored preproform of GP63 was secreted from the cell, and was the same molecular mass as the protein secreted from Δ*gpi8* cells (Naderer and McConville, 2002). The $t_{1/2}$ of secretion of this GP63 isoform was 120 minutes, the same as that found for secretion of GP63 from the Δ*gpi8* cell line (Figure 4.4).

A detailed analysis of the processing of GP63 indicated that all WT isoforms of GP63 identified in this study were GPI-anchored (Figure 4.7). The removal of the C-terminal GPI signal and GPI anchor addition do not account for any of the WT processing intermediates identified. No unanchored forms were detectable by the methods used and the immature 65c precursor detectable at time 0 minutes of chase was GPI-anchored. This demonstrates that GPI anchor addition occurs rapidly following translation and translocation of the protein into the ER. GPI anchor addition is known to occur rapidly and it has previously been shown that GPI anchors are added to awaiting VSG proteins within 2 minutes of the proteins translation (Bangs *et al.*, 1985; Ferguson *et al.*, 1986).

Treatment of cells with tunicamycin, or treatment of cell lysates with PNGaseF demonstrated that the 65c isoform present at time 0 was N-glycosylated (see figures 4.9 and 4.10). The finding that the first identifiable form of GP63 in WT cells is both GPI-anchored and N-glycosylated shows that addition of N-glycans is also rapid. The close association of the GPIT complex and the translation machinery is suggested by several recent studies. Immune-fluorescence microscopy demonstrated that in *T. brucei* GPI8 co-localised with QM, a 60S ribosomal protein (Lillico *et al.*, 2002). This indicates a close association of the GPIT with the rough ER. Mutational experiments using a wheat germ translation system supplemented with mammalian microsomes examined the processing of a GPI-anchored protein with a 43 amino acid extension at the C-terminal end (Vidugiriene *et al.*, 2001). Proteolysis was used to isolate protease-protected proteins, deemed to be membrane translocated, and suggested that the ribosome-bound

C-terminally extended protein became GPI-anchored. This implies that GPIT can access ribosome bound proteins, and is positioned close to the translocon in mammalian cells. However it was also possible that the protein was cleaved from the ribosome by proteolysis prior to anchor addition, and the results were therefore inconclusive (Vidugiriene *et al.*, 2001). Attempts to co-purify the *S. cerevisiae* GPIT complex with Sec61p, a component of the yeast translocon, or Wpb1, a component of the yeast OST, were unsuccessful (Fraering *et al.*, 2001). There is no direct evidence that GPIT associates with either the OST or the translocon, however the rapidity of anchor addition subsequent to translocation and glycosylation and the proximity of GPIT to the translocon and translational machinery strongly indicate that this is likely.

Treatment with tunicamycin or PNGase F showed that all detected cellular isoforms of GP63 from WT and Δ*gpi8* cells were N-glycosylated. The secreted form of GP63 was also confirmed to be N-glycosylated by ConA precipitation. This demonstrates that GPI-anchor addition is not required for the N-glycosylation of GP63. In WT cells treated with tunicamycin only 2 major (63ct and 60ct) and one minor isoforms of GP63 were identified, compared to the 4 isoforms of GP63 (65c, 64c 63c and the fourth minor intermediate between 65 and 64kDa) identified in WT cells grown under normal conditions. This suggested that at least one of the processing intermediates identified under normal conditions was due to the processing of N-glycans. However this hypothesis was not confirmed by PNGaseF treatment, as the 4 WT isoforms of GP63 each underwent a size shift and 4 deglycosylated GP63 isoforms were still detected (63cp, 62cp, 61cp, and 60cp). If N-glycans were processed during the trafficking of GP63, PNGaseF treatment would be expected to result in the resolution of 2 of the detected isoforms into a single sized protein. The loss of N-glycosylation affected the onward processing of GP63 in tunicamycin treated cells. PNGaseF treatment examined the N-glycosylation state of isoforms that had been processed normally in untreated cells. The different isoforms of GP63 identified in WT cells may not be caused by the processing of N-glycans, as originally hypothesised subsequent to examining the results from tunicamycin treatment, however the presence of the N-glycans may be important for the correct processing of GP63 (N-glycan mediated processing).

Loss of N-glycosylation by tunicamycin treatment effects the level of GP63 secretion. This result is particularly clear in the Δ*gpi8* cell line where the level of secretion of non-GPI-anchored GP63 is dramatically reduced (Figure 4.9, panel B). Therefore loss of N-glycosylation effects both the intracellular processing of GP63 in WT cells, and secretion of GP63 from the cell, with particular effect on the non-GPI-anchored GP63 isoform in the Δ*gpi8* cell line.

In other systems treatment of cells with tunicamycin has been shown to affect the secretion of proteins. The mammalian zinc metalloproteinase, meprin A, consists of 2 subunits; meprin α has 10 potential N-glycosylation sites and is secreted if not associated with meprin β, an integral membrane protein. Individual point mutation of any of the potential N-linked glycosylation sites of meprin α did not prevent its secretion from Madin-Darby canine kidney (MDCK) cells, however mutation of all 10 sites resulted in retention of the protein in the cell, and no secretion (Kadowaki *et al.*, 2000). Treatment of MDCK cells with tunicamycin resulted in the decrease in both the biosynthesis and secretion of the meprin α subunit. N-linked glycosylation was required for secretion of the protein from MDCK cells, and it was speculated that this was due to its requirement in the correct folding of the protein (Kadowaki *et al.*, 2000).

It has also been suggested that N-linked glycans act directly as a signal for protein trafficking. The introduction of N-linked glycosylation sites into the non-glycosylated mammalian proteins, occludin and ERGIC-53, was examined (Gut *et al.*, 1998). Occludin, is a polytopic membrane protein, and ERGIC-53 a membrane protein that cycles between the ER and Golgi. These proteins were mutated and expressed in MDCK cells and Chinese hamster ovary cells (CHO). It was shown in the absence of any other targeting signal the introduction of N-linked glycans resulted in cell surface expression. In the absence of N-glycans both chimeras accumulated within the Golgi (Gut *et al.*, 1998). N-linked glycans have multiple functions, including the correct folding and sorting of proteins in the ER through interaction with the calnexin-calreticulin pathway, and thus mediate ER-to Golgi transport (Helenius and Aebi, 2001). In the absence of other signals they appear to act as a signal for secretion and trafficking. It therefore seems possible that in the Δ*gpi8* cell line N-glycans are required for the secretion of GP63 from the cell. It would be interesting to examine the fate of

GP63 within the Δ*gpi8* tunicamycin treated cells. The loss of secretion suggests that the protein is retained or degraded intracellularly. Immune-fluorescence microscopy may determine where this degradation occurs. In WT cells N-glycans are not required for the trafficking of GPI-anchored GP63 to the cell surface. It is possible that the GPI anchor plays an active role in directing trafficking, and this point will be discussed later in this chapter.

Previous studies have examined the effects of loss of N-glycosylation on GP63 in WT cell lines. *L. major* promastigotes were grown in medium containing tunicamycin (Funk *et al.*, 1994). Biotin labelling and PI-PLC treatment was used to demonstrate that non-glycosylated GP63 was GPI-anchored on the cell surface. This protein was also shown to be active. Endo-F treatment of PI-PLC cleaved GP63 from both *L. major* and *L. mexicana* was used to demonstrate that N-glycosylation is not necessary for GP63 activity (Funk *et al.*, 1994). Removal of N-glycosylation by site directed mutagenesis at any or all of the 3 N-glycosylation sites identified in *L. major* GP63, resulted in lower levels of cellular GP63 (McGwire and Chang, 1996). This was not due to secretion from the cell, and it was suggested that N-glycans may contribute to the intracellular stability of GP63. All mutant forms of deglycosylated GP63 showed azocasein or gelatinolytic activity, and it was suggested that the activity of the deglycosylated mutants increased compared to WT GP63 (McGwire and Chang, 1996). An *L. donovani* GP63 deficient strain expressing *L. chagasi* GP63 from a plasmid, was grown in medium containing tunicamycin (Yao *et al.*, 2002). 2 isoforms of GP63 were detected in cells grown under normal conditions, and 2 GP63 isoforms of decreased size were identified from tunicamycin treated cells. Similarly treatment of cell lysate with N-glycanase demonstrated that the 2 detectable GP63 isoforms were both N-glycosylated (Yao *et al.*, 2002). These studies showed that, similar to this study, in the cell lines examined all detectable isoforms of GP63 are N-glycosylated. They also demonstrated that in the absence of N-glycans GP63 is processed and GPI-anchored on the cell surface in an active form.

A recent study has also examined the secretion of non-glycosylated GP63 (McGwire *et al.*, 2002). Expression from a plasmid of either WT and or a non-glycosylated mutant form of GP63 in a *L. amazonenesis* GP63 deficient cell line showed that GP63 was

secreted into the medium (McGwire *et al.*, 2002). Both the re-expressed WT and deglycosylated forms were released into the medium at similar levels and with the same kinetics, and it was concluded that N-glycosylation does not effect the extracellular release of GP63 (McGwire *et al.*, 2002). The study did not address the intracellular processing of the non-glycosylated GP63, neither did it examine the secretion of non-glycosylated, non-GPI-anchored GP63. It would be interesting to express a mutant form of GP63 which is neither N-glycosylated, nor GPI-anchored using the *L. amazonensis* system as this would allow direct comparison with Δ*gpi8* cells grown in tunicamycin.

PNGase F treatment of pulse-chase labelled cell lysates, resulted in the separation of the 65c isoform present after time 0 minutes of chase in both WT and Δ*gpi8* cells, into a 63kDa and a 62kDa doublet (Figure 4.10). This suggests that the unprocessed 65kDa protein may consist of 2 isoforms of GP63. In the WT cells both isoforms appear to be processed to the 63c isoform, whilst in the Δ*gpi8* cell line both isoforms remain unprocessed. However treatment of cells with tunicamycin did not produce a similar result, this was most clearly seen in the Δ*gpi8* cell line where only a single 63ct isoform could be detected subsequent to treatment with tunicamycin (Figure 4.9). In a recent study 3 isoforms of GP63 were detected during pulse-chase labelling of WT *L. mexicana*. It was suggested that the 64kDa (P2) isoform was comprised of 2 subsets of GP63, a processed form of the 65kDa proform, and a second major pro-form of GP63, (Ralton *et al.*, 2002). Pulse-chase labelling experiments were performed on the *L. mexicana* DIG2 mutant cell line, which has a defect in both GIPL and GPI-anchor biosynthesis and secretes unanchored GP63 (Naderer and McConville, 2002). Two different sized isoforms of GP63 were synthesised, as detected by SDS-PAGE. A large proportion of GP63 was secreted, however immune-fluorescence microscopy also suggested that a sub-population of GP63 was retained within the ER where it was thought to be degraded. It was suggested that the two isoforms of GP63 had differing fates within the mutant cell line (Naderer and McConville, 2002).

In this study 20% less GP63 was detected in the Δ*gpi8* cell line compared to WT, as assessed by the total levels of GP63 present in the medium and cellular fractions (Figure 4.6). It is possible that this difference was due to the intracellular degradation of

a sub-population of GP63 in the Δ*gpi8* cell line. Northern blotting experiments would allow an assessment of the levels of GP63 expression in both WT and Δ*gpi8* cells.

Activation of GP63 occurs intracellularly by the removal of the pro-region. Expression of GP63 with a mutation at the anchor addition site using a baculovirus expression system suggested that the unanchored secreted form of GP63 was inactive, (Macdonald *et al.*, 1995). Expression of GP63 mutated at the GPI anchor addition site from a vector in the *L. amazonensis* 'GP63 deficient' strain, suggested that the secreted protein had gelatinolytic activity, and was therefore thought to be active (McGwire and Chang, 1996). Though the 'GP63 deficient' cell line expresses WT GP63 at reduced levels (Kink and Chang, 1988). This study demonstrated that GP63 secreted from the WT cell line is active, though it was not investigated if this activity corresponds to only one or both of the two isoforms known to be secreted from these cells. It is not clear from the work presented in this thesis whether GP63 secreted from the *L. mexicana* Δ*gpi8* cell line is active. The difficulty in detecting active GP63 in the medium of the Δ*gpi8* cell line suggests that non-GPI-anchored GP63 is not activated during processing. However to conclusively demonstrate this an alternate approach is required. The Δ*gpi8* cell line does provide a useful tool for examining the activation of non-GPI-anchored GP63 without a background of fully processed WT protein.

Treatment of inactive secreted GP63 with $HgCl_2$, resulted in the identification of several different intermediates of GP63 as the protein underwent proteolytic degradation as it was activated (McMaster *et al.*, 1994). It is possible that pro-region cleavage is not a single event, and may therefore account for more than one of the processing intermediates detected in the WT cell line.

It is interesting that the inhibitor Brefeldin A had no effect on the processing or secretion of GP63 (Figure 4.16). BFA is an inhibitor of guanine nucleotide exchange factors (GEFs), which stimulate the activation of ARF proteins, which in turn regulate the formation of COPI vesicles, required for retrograde transport from the Golgi to the ER (Chardin and McCormick, 1999). In mammalian cells and mutant yeast cells with a permeable cell wall, treatment with BFA causes the collapse of the Golgi complex, and inhibits secretion (Peyroche *et al.*, 1999). Treatment of *T. cruzi* epimastigotes has also been shown to effect the ultrastructure of the Golgi by causing an increase in the

number and size of cisternae (Engel *et al.*, 1998). However it was not clear if retrograde protein trafficking had been inhibited in these cells. In another study on *L. mexicana* treatment of promastigotes with BFA, ilimaquinone, or monensin, all of which inhibit vesicular transport in other eukaryotes, had no effect on the rate of transport of GP63 or GIPLs, confirming the results found in this study (Ralton *et al.*, 2002). In yeast and mammalian cells BFA inhibits only a small sub-population of the GEF proteins (Chardin and McCormick, 1999; Peyroche *et al.*, 1999). It therefore seems possible that homologues to these specific GEF proteins differ, or are absent in *Leishmania*. Such differences in vesicular trafficking components between mammalian and protozoa could provide important leads in the search for new drug targets.

Examination of the secretion of GP63 provided several interesting results. Firstly WT cells secreted two forms of GP63, which during pulse-chase labelling experiments appeared in the medium at different time points (Figure 4.3, panel C). TX-114 fractionation partitioned the 65s from into the soluble fraction, whilst the 63s form partitioned into the membrane associated fraction. Subsequent to PI-PLC treatment, the 63s form could be identified within the soluble fractionation (Figure 4.15). This suggested that the 65s form was secreted without a GPI anchor and the 63s form was released with a GPI anchor. I originally thought that the 63s form was released into the medium from the cell surface by cleavage of the GPI anchor, the mechanisms for the release of GP63 from the cell surface with an intact anchor are not clear. Ethanolamine labelling followed by immune-precipitation of GP63 from the medium, or use of the CRD antibody subsequent to PI-PLC treatment of the secreted samples, would provide a clearer indication as to whether the 63s form is released into the medium with an intact anchor. The CRD antibody could also be used to assess if either of the 63s, or 65s isoforms had contained a GPI-anchor, which had subsequently been removed prior to secretion. Analysis of the intracellular fractionation of GP63 in WT cells suggested that a small proportion of GP63 was present in the soluble fraction, and the quantity was directly comparable to the level of GP63 secreted (Figure 4.5).

Analysis of the Δ*gpi8* cell line demonstrated that the level of secretion of GP63 from these cells was far higher than from the WT cell line. Indeed secretion of GP63 from WT cells was only 13% of that from Δ*gpi8* (Figure 4.4). Pulse-chase labelling

experiments indicated that the Δ*gpi8*-secreted isoform of GP63 could be detected in the medium at the 20 minute time point, the same time at which the 65s isoform from WT cells was first detected. To compare in more detail the secretion of GP63 in Δ*gpi8* cells with trafficking within WT cells, it would be useful to calculate the time it takes to traffic GPI-anchored GP63 to the surface of WT cells. It would also be interesting to compare both cell surface trafficking in WT cells, and secretion form Δ*gpi8* cells with the timing with which both the 65s, and 63s isoforms are released into the medium of WT cells. Preliminary attempts to biotin label pulse-chase labelled cells were unsuccessful, and due to time constraints it was not possible to continue this work, however this seems an interesting area for further investigation.

Collectively these results suggest that within WT *L. mexicana* GP63 is trafficked via two separate pathways, or different compartments within the same pathway (Figure 4.16). GPI-anchored GP63 is trafficked via a classical pathway whereby GP63 is N-glycosylated, GPI-anchored and then undergoes further modification during transport to the cell surface. Unanchored GP63 is trafficked via a direct secretion pathway whereby non-GPI-anchored GP63 is rapidly N-glycosylated and secreted from the cell without subsequent modification. The Δ*gpi8* cells utilise the second pathway; all GP63 is N-glycosylated and transported from the cell. The GPI-anchor therefore appears to be important in directing proteins into the first pathway. The inhibition of secretion from the Δ*gpi8* cells in the presence of tunicamycin suggests that N-glycans may play a role in the entry of proteins into the direct secretion pathway.

Work on *L. chagasi* has also identified a secreted form of GP63 from WT cells (Yao *et al.*, 2002). Only a single 63kDa form was identified, and this protein was not recognised by the CRD antibody suggesting it was not released from the cell surface by cleavage of the GPI anchor. The 63kDa protein could be fractionated into both membrane associated and soluble forms. The absence of cytosolic proteins in the medium suggested the protein was not released due to cell lysis. It was speculated that GP63 was released as both a soluble form in micelles, and also in membrane-bound vesicles (Yao *et al.*, 2002). GP63 has also been demonstrated to be secreted from both laboratory strains, and clinical isolates of *L. amazonensis*, demonstrating that the extracellular release of GP63 occurs in a WT setting (McGwire *et al.*, 2002). Labelling

experiments of *L. amazonensis* with both [S^{35}] cysteine/ methionine and biotin, suggested that the extra-cellular release of GP63 was from two separate pools, which each secreted GP63 with differing kinetics (McGwire *et al.*, 2002). Use of either the CRD antibody or ethanolamine labelling suggested the released form of GP63 was not GPI-anchored. This results differs from those found in this study on *L. mexicana* WT cells, or work on *L. chagasi* WT cells (Yao *et al.*, 2002), and may reflect differences between *Leishmania* species. The *L. mexicana* DIG1 cell line has also been shown to secrete high levels of GP63 from the cell (Naderer and McConville, 2002). Collectively this work demonstrates that different GP63 isoforms are secreted at a low level from WT cells, whilst loss of anchoring leads to a high level of secretion. The secretion of WT GP63 from promastigote cells may have a biological significance. It is possible that the extracellular release of the protease aids invasion of host cells. It has been suggested that secreted GP63 plays a role in the degradation of the extra cellular matrix after inoculation into the host by the sandfly (McGwire *et al.*, 2002). It is also possible that the secreted protein aids the evasion of complement mediated lysis, similar to the function of membrane anchored GP63 (Brittingham and Mosser, 1996).

The fate of misprocessed GPI-anchored proteins has been examined within other systems. In mammalian cells a reporter protein with a mutation at the site of GPI anchor addition (hGHDAF28) was shown to be retained and degraded within a pre-Golgi compartment (Moran and Caras, 1992; Field *et al.*, 1994). The use of yeast cells deficient in GPI anchor biosynthesis due to inositol starvation, demonstrated that GPI-anchored proteins were incorporated into ER transport vesicles and GPI-anchor attachment was a requirement for this to occur (Doering and Schekman, 1996). It is thought that non-GPI-anchored proteins are prevented from entering the secretory pathway, to avoid competition with correctly processed proteins. The precise mechanism for the ER retention and subsequent degradation of non-GPI-anchored proteins is not known. It is possible that the misprocessed proteins enter the calnexin/ calreticulin system which modulates the forward trafficking of glycoproteins (Parodi, 2000; Ellgaard and Helenius, 2001), or are directed from the ER via the translocon pore for cytoplasmic degradation (Wiertz *et al.*, 1996; Wilbourn *et al.*, 1998; Ali *et al.*, 2000). Mutation of a single cysteine residue present within the C-terminal GPI signal of the reporter protein hGHDAF28, (also mutated at the site of GPI anchor addition

preventing GPI anchor addition), resulted in the secretion of the fusion protein instead of ER retention (Wilbourn *et al.*, 1998). It was therefore suggested that ER quality control mechanisms actively retain non-GPI-anchored proteins.

Expression of VSG in procyclic *T. brucei* demonstrated that mutation of the VSG anchor addition site resulted in the ER retention of the protein, and extracellular release with highly reduced kinetics (McDowell *et al.*, 1998). Studies in bloodstream form *T. brucei* expressed mutated forms of VSG-221 against a VSG-117 (WT) background. Truncation of VSG-221 to delete the entire C-terminal signal sequence, resulted in retention of the protein in the ER as determined by immune-fluorescence, and co-localisation with the ER protein BiP (Bohme and Cross, 2002). The mutated VSG was determined to be correctly folded and dimerised. Replacement of the C-terminal signal with a transmembrane domain did not produce cell surface expression, instead the protein was retained intracellularly close to the flagellar pocket in possible pre-lysosomal compartments (Bohme and Cross, 2002). Expression of GPI-PLC in *T. cruzi* resulted in the depletion of GPI proteins from the cell surface, and depletion of GPI protein intermediates. A dominant negative effect resulted in a loss of a functional GPI-biosynthetic pathway (Garg *et al.*, 1997). Within these cells the fate of four different GPI-anchored proteins was examined, two were secreted into the medium and two were degraded intracellularly (Garg *et al.*, 1997). It was concluded that GPI-anchors were required for the correct surface expression of proteins, and the fate of the misprocessed GPI protein depended on the protein's structure (Garg *et al.*, 1997; Bohme and Cross, 2002). It has been speculated that the ER retention of non-GPI-anchored proteins may be due to sustained interaction with the GPIT complex (Garg *et al.*, 1997; Bohme and Cross, 2002).

The fate of non-GPI-anchored proteins was also investigated in the *T. brucei* mutant strain, GPI10, defective in the addition of the third mannose to the GPI backbone (Nagamune *et al.*, 2000). In 'GPI-sufficient' cells EP procyclin (the major GPI-anchored protein of procyclic trypanosomes) was produced as 35, 40 and 50kDa peptides, and chased into a mature 50kDa form. A small amount of this mature form was secreted. In the GPI deficient cells, mature 50kDa procyclin was not produced. Several smaller peptides were produced, and smaller peptides were also detected in the

medium. It was concluded that non-GPI-anchored EP procyclins were secreted into the medium, and degraded, and loss of GPI anchoring affected the processing of the protein (Nagamune *et al.*, 2000).

These studies collectively suggest that two parallel processes may affect the fate of GPI-anchored proteins. The first is the active retention of misprocessed proteins within the ER, the second is the active requirement for a GPI anchor for forward transport. It is possible that *Leishmania* and Trypanosomes lack the complex quality control mechanisms developed in higher eukaryotes for the intracellular retention and degradation of misprocessed proteins, though homologues to calnexin have been identified in *L. donovani* and *T. cruzi* (Joshi *et al.*, 1996; Labriola *et al.*, 1999).

GPI-anchors have recently been associated with the trafficking of proteins via interactions with lipid microdomains, liquid-ordered regions enriched in cholesterol and sphingolipids, termed lipid rafts (Simons and Ikonen, 1997; Muñiz and Riezman, 2000; Ikonen, 2001). This suggests a process in which the GPI-anchor is directly required for the forward transport of proteins.

Studies of mammalian cells demonstrate that GPI-anchored proteins become insoluble to detergent extraction during trafficking through the secretory pathway and form detergent resistance membranes (DRMs), enriched in sterols, sphingolipids, glycosphingolipids and GPI-anchored proteins (Brown and Rose, 1992). This incorporation of GPI-anchored proteins into lipid rafts in the Golgi of mammalian cells (Brown and Rose, 1992; Simons and Ikonen, 1997). The depletion of ceramide or cholesterol in MDCK cells specifically affects the sorting and trafficking of GPI-anchored proteins to the cell surface. This suggests that the association of GPI-anchored proteins with lipid rafts is required for the delivery of proteins to the cell membrane (Mays *et al.*, 1995; Hannan and Edidin, 1996). It has also been shown that though a GPI-anchor is required to mediate raft association within MDCK cells, it is not sufficient for the correct targeting of a protein to the cells apical surface (Benting *et al.*, 1999)

The specific association of GPI-anchored proteins with lipid rafts, and their requirement in the delivery of these proteins to the plasma membrane has also been

demonstrated in yeast. Yeast cells depleted in ceramide by growth in the inhibitor myrocin, showed a rapid reduction in GPI-protein transport, whilst the maturation and transport of both soluble and transmembrane proteins was unaffected (Horvath *et al.*, 1994). Similarly a ceramide bio-synthesis deficient cell line was unable to transport the GPI-protein Gas1p to the Golgi, but secretion of a soluble protein was not abolished. (Sütterlin *et al.*, 1997; Bagnat *et al.*, 2000). DRMs were shown to be present in the yeast ER, suggesting a difference in the location of raft formation in mammalian and yeast cells, and demonstrating that GPI-proteins are sorted from other proteins at an early stage of the secretory pathway (Muñiz and Riezman, 2000; Bagnat *et al.*, 2000) .

In *T. brucei* a novel bloodstream stage alanine-rich protein (BARP), predicted to be GPI-anchored, localised in a punctate pattern on the cell surface (Nolan *et al.*, 2000). This protein could be fractionated into a detergent insoluble, glycolipid-enriched fraction by cold TritonX-100 extraction, while the predominant GPI-anchored protein VSG did not. It was suggested that this was the first example of lipid-microdomains in protozoa (Nolan *et al.*, 2000).

DRMs have also been isolated from *L. major*, and are enriched in characteristic eukaryotic lipid raft components; inositol phosphorylceramide (IPC), sterol (ergosterol), and GPI-anchored molecules (both GP63, and LPG) (Denny *et al.*, 2001). GP63 is rapidly incorporated into DRMs suggesting that rafts may form in the ER of *Leishmania* as in yeast (Denny *et al.*, 2001). In *L. mexicana* both GIPLs and GP63 have been demonstrated to associate into TX-100 insoluble membranes (Ralton *et al.*, 2002). These insoluble membranes have similar characteristics to DRMs from other eukaryotes, and are enriched in IPC (the major sphingolipid of *L. mexicana*), but depleted in glycerophospholipids, and cellular proteins. Pulse-chase labelling, and TX-100 fractionation demonstrated that the major isoform of GP63, was both transported to the cell surface, and incorporated into DRMs with similar kinetics, and that GP63 was incorporated into DRMs in a late secretory compartment in *L. mexicana*. This is different from the results from the *L. major* study whereby it was suggested that DRM incorporation occurred in an early secretory compartment (Denny *et al.*, 2001).

Low temperatures prevent the transport vesicles which bud from the ER from fusing with the Golgi, and therefore blocks secretory transport. In *T. brucei* the transport of

VSG from the Golgi to the flagellar pocket is inhibited at low temperatures (Duszenko *et al.*, 1988), similarly in mammalian cells the transport of free GPIs to the cell surface is inhibited at 15°C (Baumann *et al.*, 2000). In *L. mexicana* GP63 and GIPL incorporation into DRMs was inhibited at low temperatures, and it is suggested that this is evidence for a vesicular transport mechanism (Ralton *et al.*, 2002). It was also demonstrated in *L. mexicana* that in the presence of the sphingolipid biosynthesis inhibitor, myrocin, sphingosine and ceramide synthesis were completely inhibited. However GP63 surface transport, and GP63 and GIPL incorporation into DRMs was not inhibited. This is in contrast to the situation in other eukaryotes where the transport of GPI-anchored proteins requires the ongoing synthesis of sphingolipids and ceramides (Ralton *et al.*, 2002).

It therefore seems that GPI anchors play an important role in the forward transport of proteins in both higher eukaryotes, and protozoa, providing an interesting area for further study. Variations in this process between mammalian cells, yeast and protozoa have already been detected. Continued study using the Δ*gpi8* cell line would provide an interesting control in the study of trafficking of GPI-anchored proteins within *Leishmania*. It would be interesting to use ergosterol inhibitors to prevent raft formation in the Δ*gpi8* cell line, and compare the processing and trafficking of GP63 in these cells with the situation in WT cells.

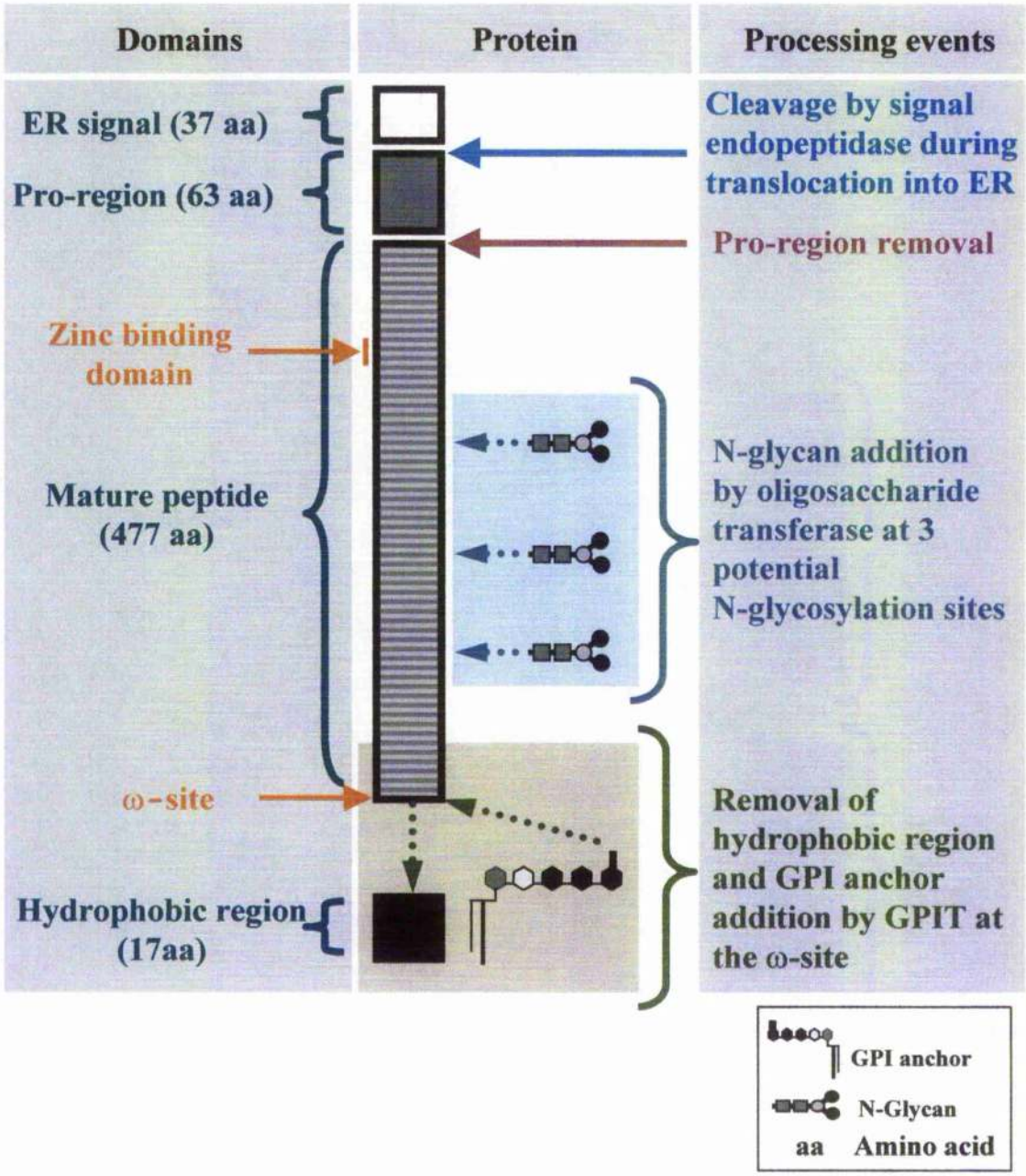


Figure 4.1: Characteristic domains of GP63, a GPI-anchored zinc metalloproteinase. GPI-anchored proteins have 4 characteristic domains; an N-Terminal ER signal peptide, mature peptide and C-terminal hydrophobic tail. The ω-site adjacent to the hydrophobic tail is the point of GPI anchor addition. Metalloproteinases are characterised by a pro-region and zinc-binding domain. GP63s from a number of *Leishmania* species are predicted to have 3 N-glycosylation sites.

Figure 4.2: Amino Acid sequence alignment of *Leishmania* GP63 homologues.
The predicted protein sequence of a *L. mexicana* GPI-anchored GP63 (L.mex), aligned with *L. major* (L.maj) accession number P08148, and *L. amazonensis* (L.ama) accession number L46798 GP63s. The alignment was performed using Align X (InforMax. Inc). Identical residues are shaded pink, conserved and similar residues are shaded grey. Weakly similar residues are shown in blue.

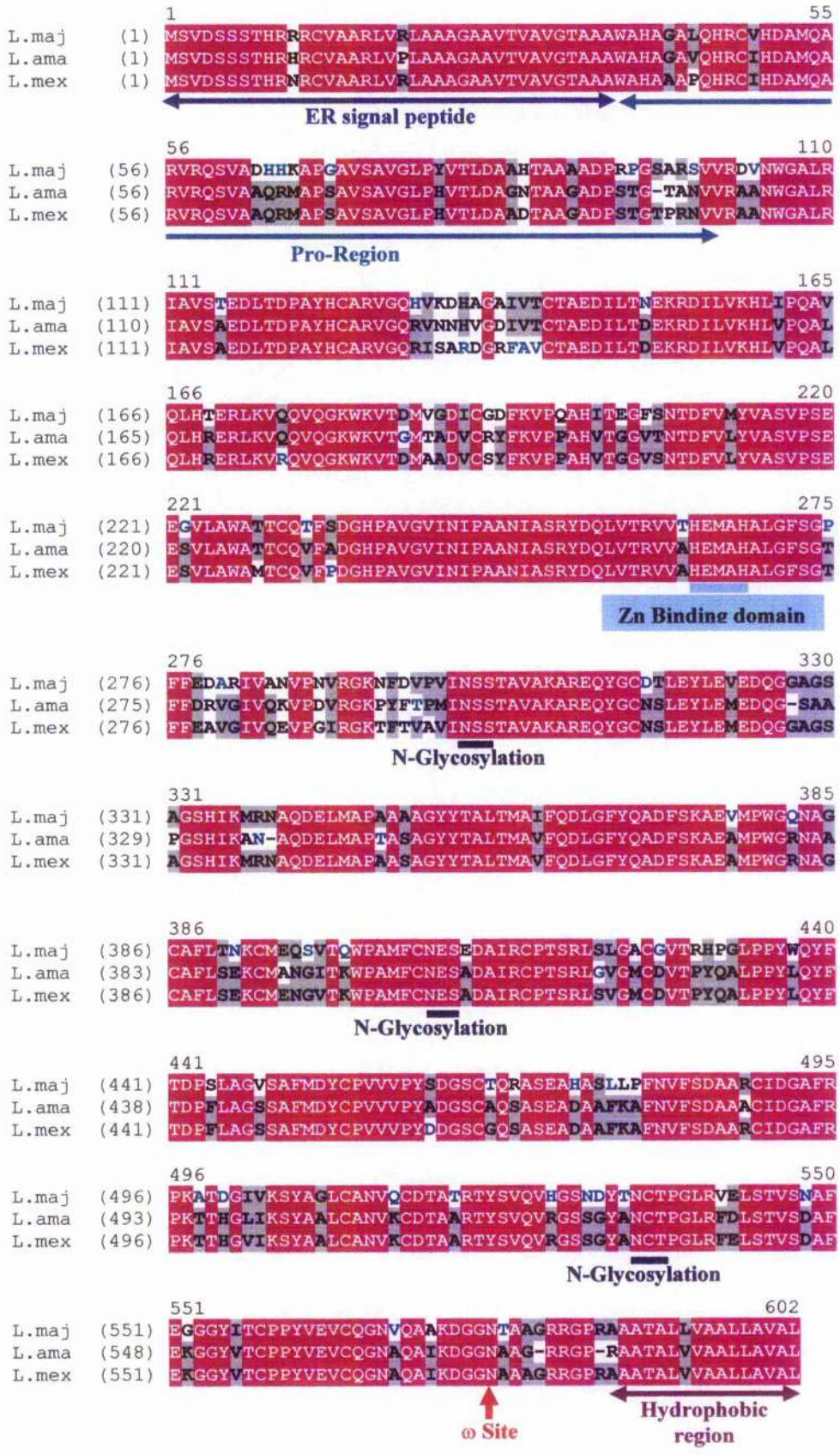

ER signal peptide
Pro-Region
Zn Binding domain
N-Glycosylation
N-Glycosylation
N-Glycosylation
ω Site
Hydrophobic region

Figure 4.3: Analysis of the intracellular processing of GP63 by pulse chase labelling.

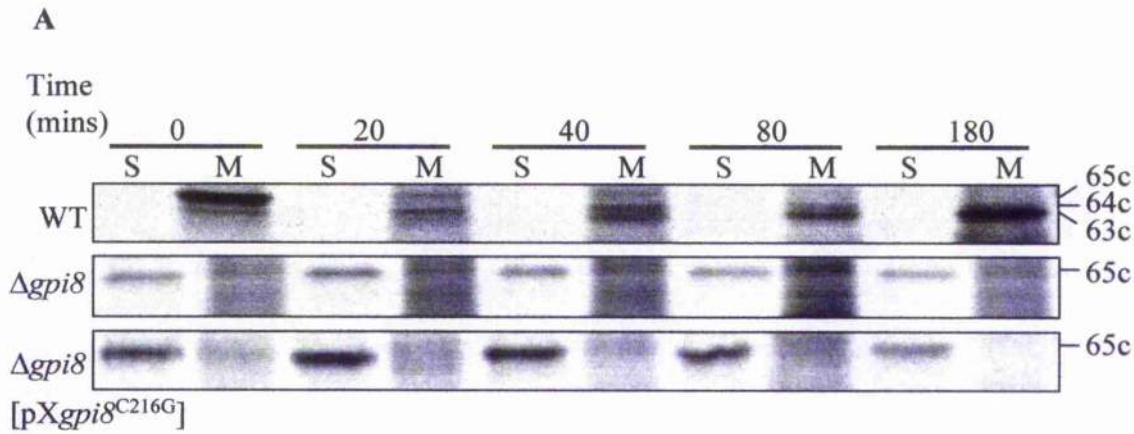
WT, Δ*gpi8* and Δ*gpi8*[pXΔ*gpi8*^{C216G}] cells were grown to mid log phase, and labelled in medium containing ^{35}S-ExpreSS for 12 minutes, washed 3 times and resuspended in an equivalent volume of growth medium. The chase time was for a period of up to 300 minutes. Equivalent samples of cells and medium were collected at suitable time points.

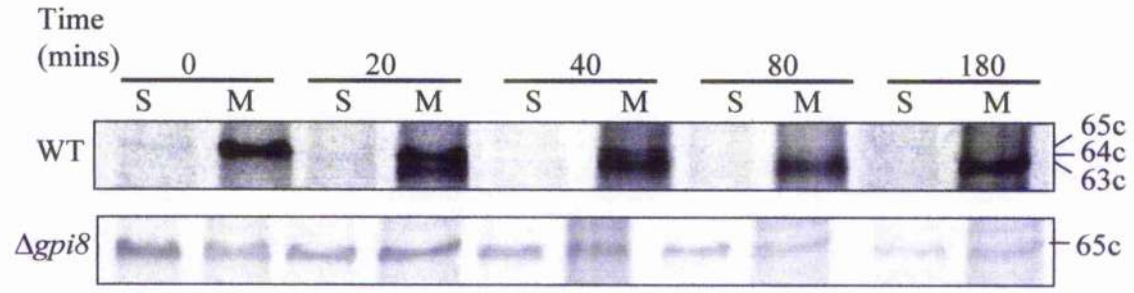
Panel A) Cell samples were partioned into soluble (S) and membrane-bound (M) fractions by TX-114 extraction.

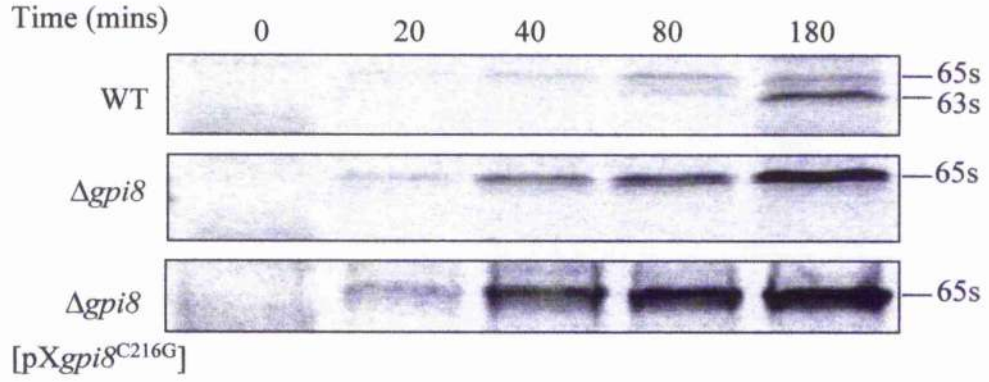
Panel B) Cell samples were partitioned into soluble and membrane-bound fractions by $NaCO_3$ extraction.

Panel C) Medium samples.

All samples were immune-precipitated with an α-GP63 antibody (L3.8), electrophoresed on a 12% SDS-PAGE gel and scanned with a phosphor-imager. Secreted (s) and cellular (c) GP63 are indicated.


A
Time (mins)
0
20
40
80
180
S
M
WT
Δgpi8
Δgpi8
[pXgpi8C216G]
65c
64c
63c
65c
65c


B
Time (mins)
0
20
40
80
180
S
M
WT
Δgpi8
65c
64c
63c
65c


C
Time (mins)
0
20
40
80
180
WT
Δgpi8
Δgpi8
[pXgpi8C216G]
65s
63s
65s
65s

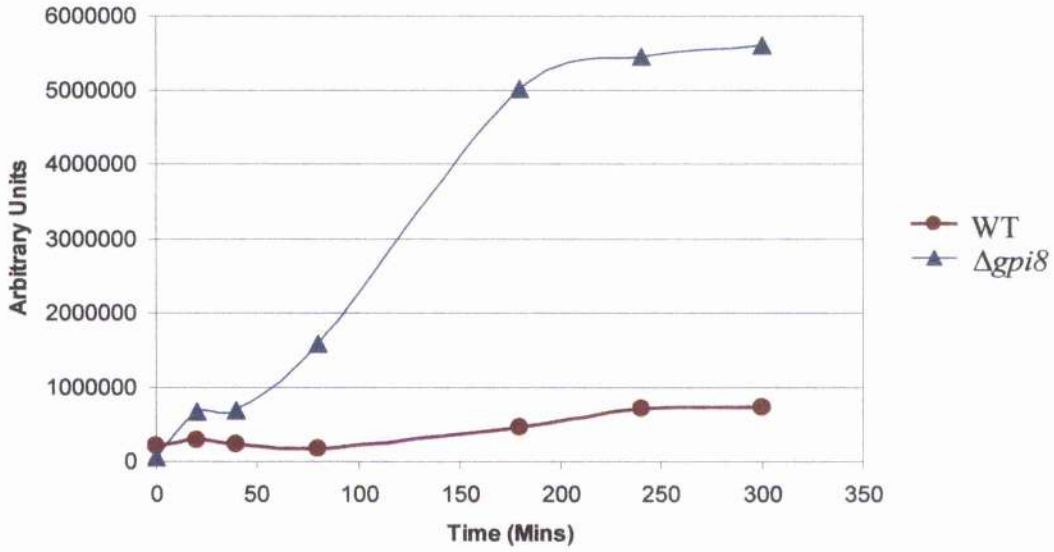


Figure 4.4: Kinetics of GP63 secretion from the cell.

The comparative levels of GP63 secreted into the medium from WT (●) and Δ*gpi8* (▲) cells during a 300-minutes pulse-chase experiment were measured using the phosphor-imager and quantified using the Image Quant programme (Molecular Dynamics). This data is from a single experiment, and is representative of a number of repeated experiments. The experiment was repeated 4 times to 180 minutes, and twice to 300 minutes.

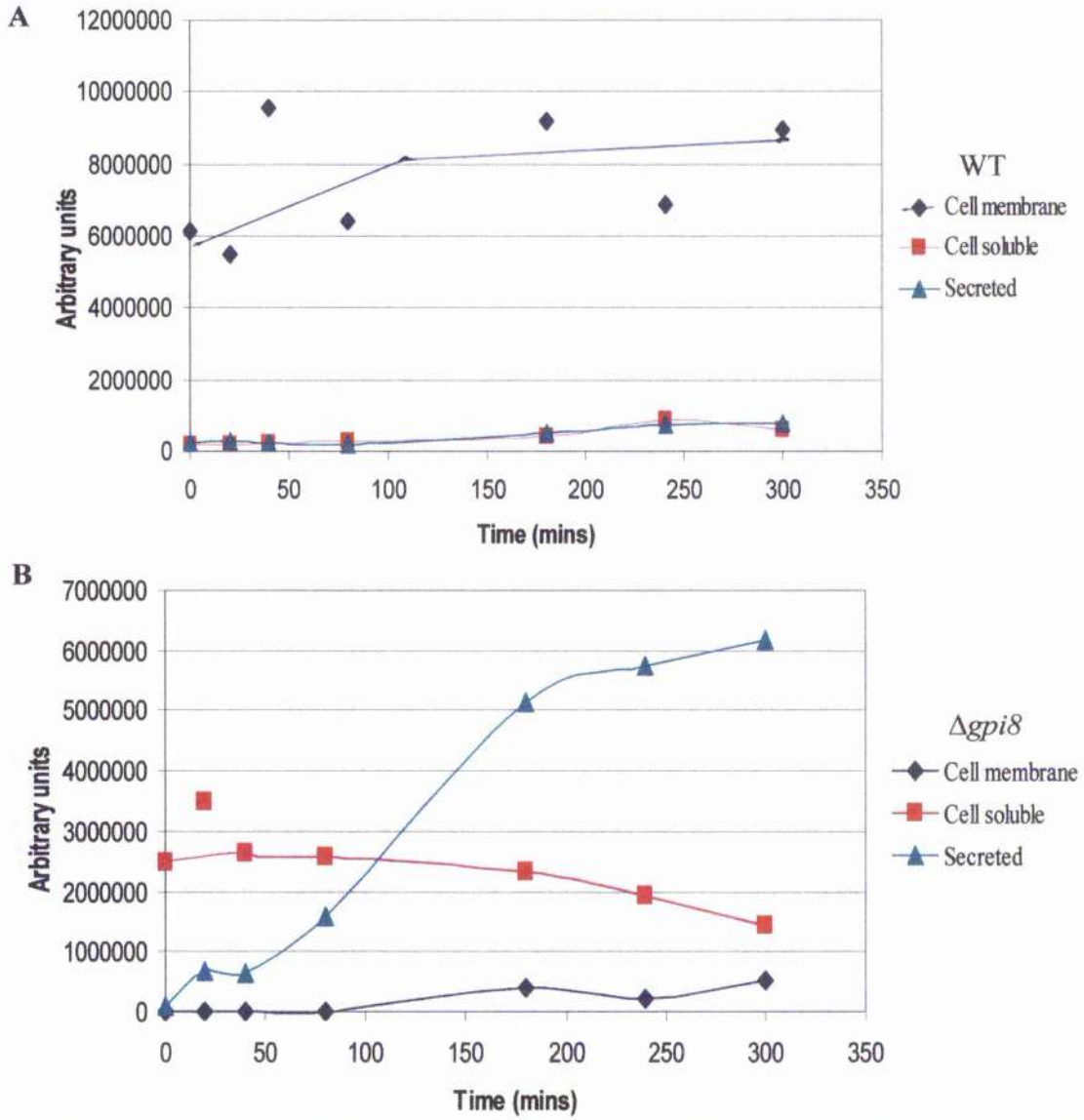


Figure 4.5: Comparative distribution of GP63 within WT and Δ*gpi8* cells over a 300 minute chase period.

Cells were pulse-chase labelled and treated as previously described. The level of membrane associated (◆), cell soluble (■) and secreted (▲) GP63 was measured and quantified using the Typhoon phosphorimager, and Image Quant programme. **Panel A** shows the distibution of GP63 in WT cells. **Panel B** shows the distribution of GP63 in Δ*gpi8* cells. The data is from one experiment, and the levels of GP63 within and between both cell lines is directly comparable. This data is representative of a number of repeated experiments. The experiment was repeated 4 times to 180 minutes, and twice to 300 minutes.

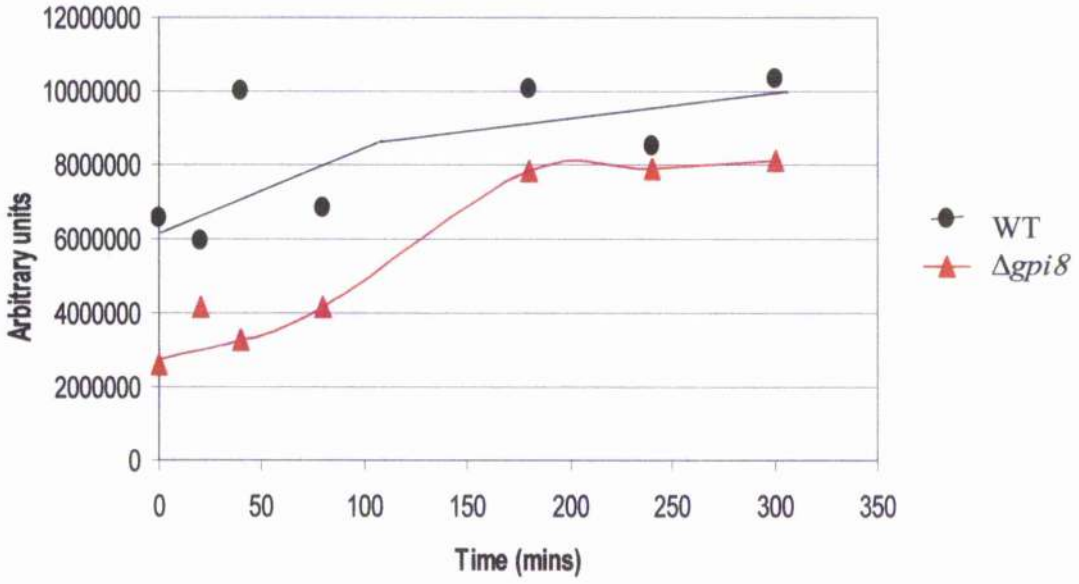


Figure 4.6: Total GP63 expression within WT and Δ*gpi8* cells.

Graph of relative GP63 expression from WT (●) and Δ*gpi8* (▲) cells. The figures were calculated from the combined fractions collected during a 300 minute pulse-chase labelling experiment. This data is representative of a number of repeated experiments. The experiment was repeated 4 times to 180 minutes, and twice to 300 minutes.

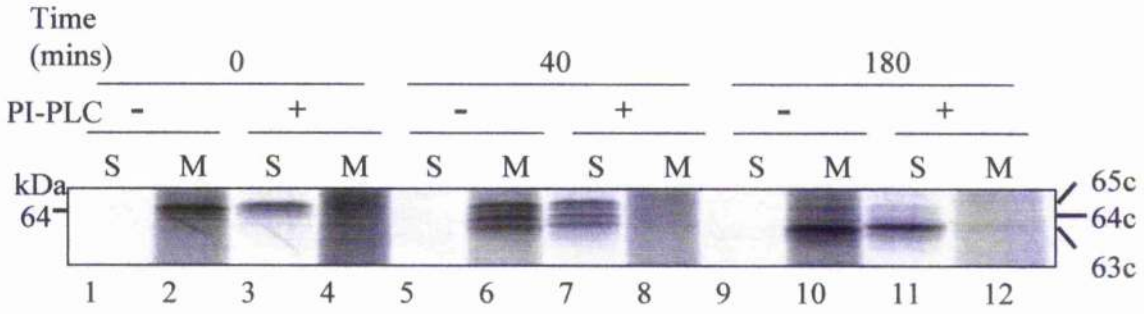


Figure 4.7: PI-PLC treatment of cells.

WT promastigotes were pulse chase labelled with ^{35}S-ExpreSS for a period up to 180 minutes. The cells were lysed and treated with or without PI-PLC prior to Triton X-114 fractionation into soluble (S) and membrane-associated (M) fractions, immune-precipitated with an α-GP63 antibody (L3.8) and electrophoresed on a 12% PAGE gel. The gel was visualised with a phosphor-imager.

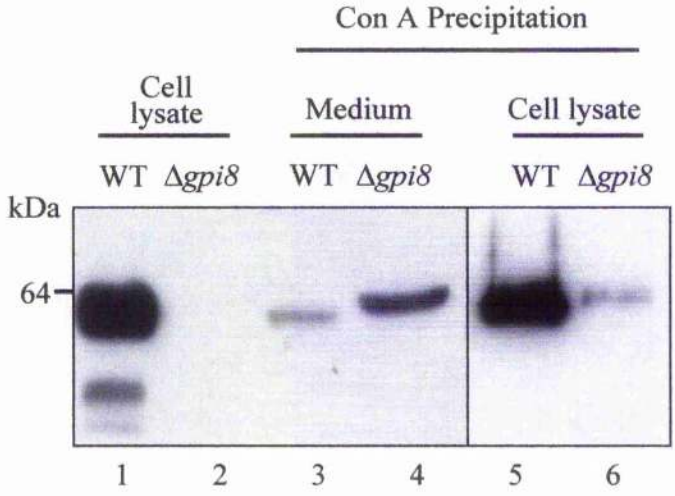


Figure 4.8: Glycosylation demonstrated by Con A precipitation.

Proteins were precipitated using ConA sepharose beads from either medium or cell lysates in two separate experiments and electrophoresed on a 12% SDS gel. Equivalent WT and Δ*gpi8* samples were loaded from either the medium (Lanes 3 and 4) or cell lysate (Lanes 5 and 6) ConA precipitation experiments, allowing direct comparison between the two cell lines. Untreated cell lysate material was loaded as a control (Lanes 1 and 2). The gel was electroblotted and GP63 detected by western blotting using a GP63 antibody.

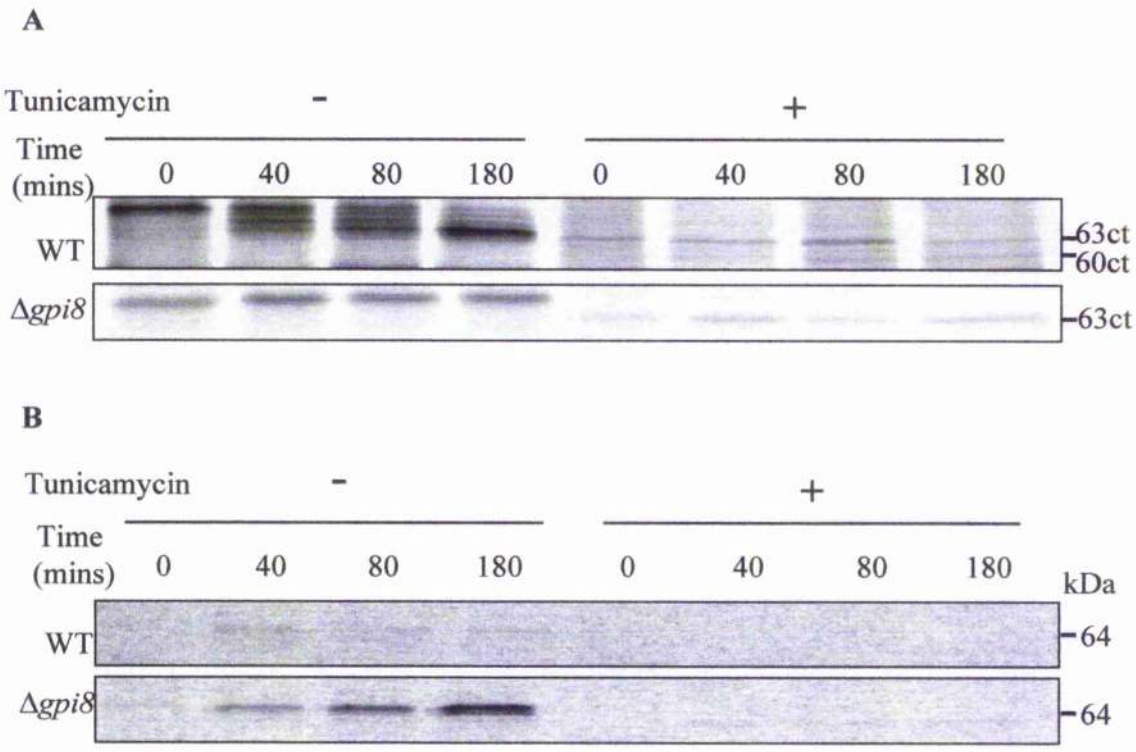


Figure 4.9: Tunicamycin treatment of cells.

Cells were grown in the presence or absence of 5 μg ml^{-1} tunicamycin for 5 hours. Cells were labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 180 minutes. Samples were taken for analysis at appropriate time points.

Panel A) Cell samples were TX-114 fractionated, immune-precipitated with a GP63 antibody, and electrophoresed on a 12% SDS gel. Only the membrane fractions from the WT cells, and the soluble fractions from the Δ*gpi8* cell line are shown here, as these were the fractions which contained detectable GP63. The different sized forms present when cells were grown in the presence of tunicamycin are indicated (ct).

Panel B) Medium fractions were immune-precipitated with an antiGP63 antibody and electrophoresed on a 12% SDS PAGE gel.

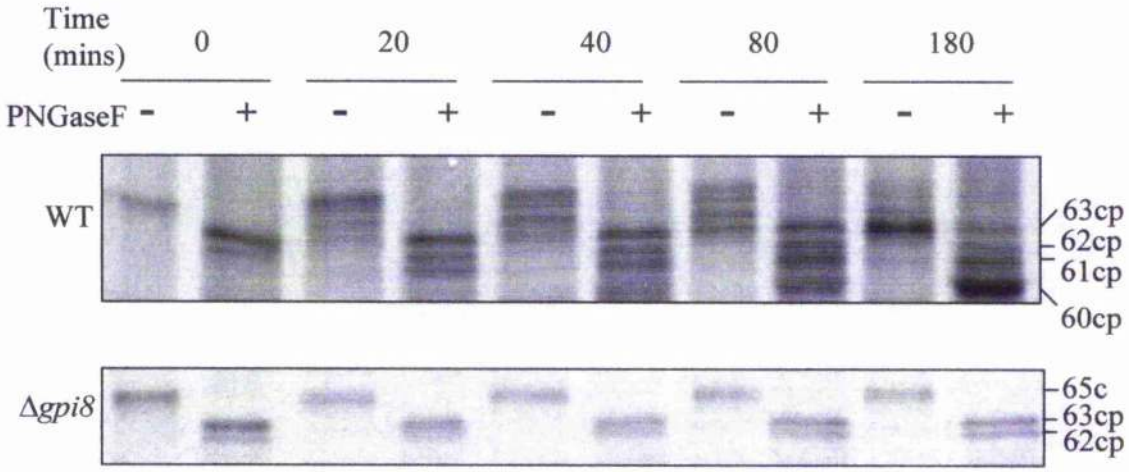


Figure 4.10: PNGase F treatment of WT and Δ*gpi8* isoforms of GP63.

WT and Δ*gpi8* promastigotes were labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 180 minutes. Samples were collected at appropriate time points, TX-114 fractionated, and immune-precipitated with an αGP63 antibody. For each sample half was treated with PNGaseF, and half mock treated. The membrane fractions from WT cells, and the soluble fractions from the Δ*gpi8* cells are shown here.

The different sized isoforms present subsequent to PNGaseF treatment are indicated (cp).

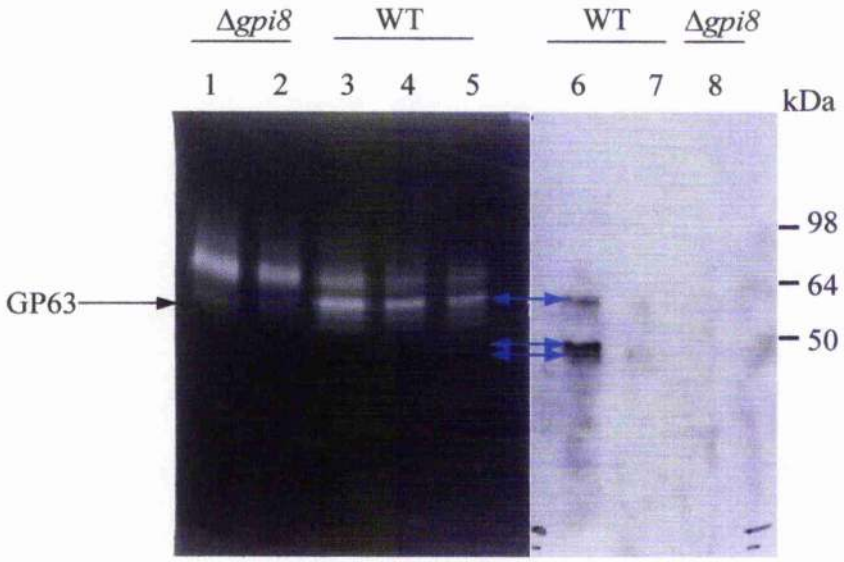


Figure 4.11: Comparison of the gelatinolytic activity of GP63 from cell lysate material.

WT and Δ*gpi8* cells were grown to late log phase. Differing cell equivalents were loaded on a 10% acrylamide 0.1% gelatin gel; $5x10^6$ cell equivalents lanes 1, 3, 6, and 8; $2x10^6$ cell equivalents lanes 2, 4, and 7; and $1x10^6$ cell equivalents lane 5. Subsequent to electrophoresis, the gel was cut in 2 and one section electroblotted and GP63 detected by western blotting. The other section was treated for gelatinolytic activity.

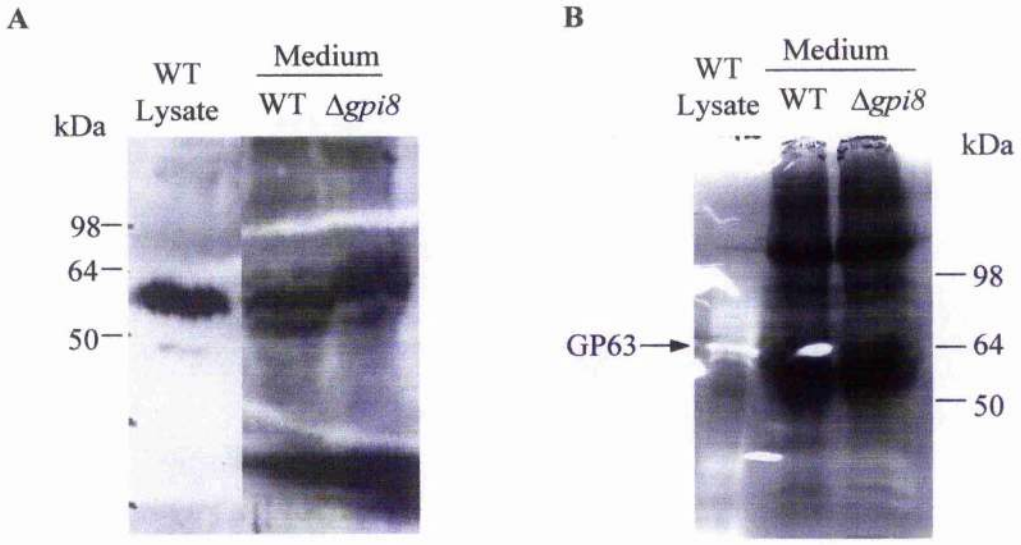


Figure 4.12: Gelatinolytic activity of proteins precipitated with ConA from the medium of WT and Δ*gpi8* cells.

WT and Δ*gpi8* cells were grown to late log phase in medium containing 5% FCS. The medium from $5x10^8$ cells was collected, concentrated and ConA precipitated.

Panel A) WT cell lysate from $1x10^7$ cell equivalents, and ConA precipitated samples from the medium of an estimated $1x10^8$ cells were electrophoresed by 12% SDS-PAGE, electroblotted and GP63 detected by western blotting.

Panel B) WT cell lysate from $1x10^7$ cell equivalents, and ConA precipitated samples from the medium of an estimated $2x10^8$ cells were electrophoresed on a 10% SDS-PAGE gel containing 0.1% gelatin. The gel was treated for gelatinolytic activity by incubating in 0.1M Na Acetate, 1mM DTT at 37°C overnight.

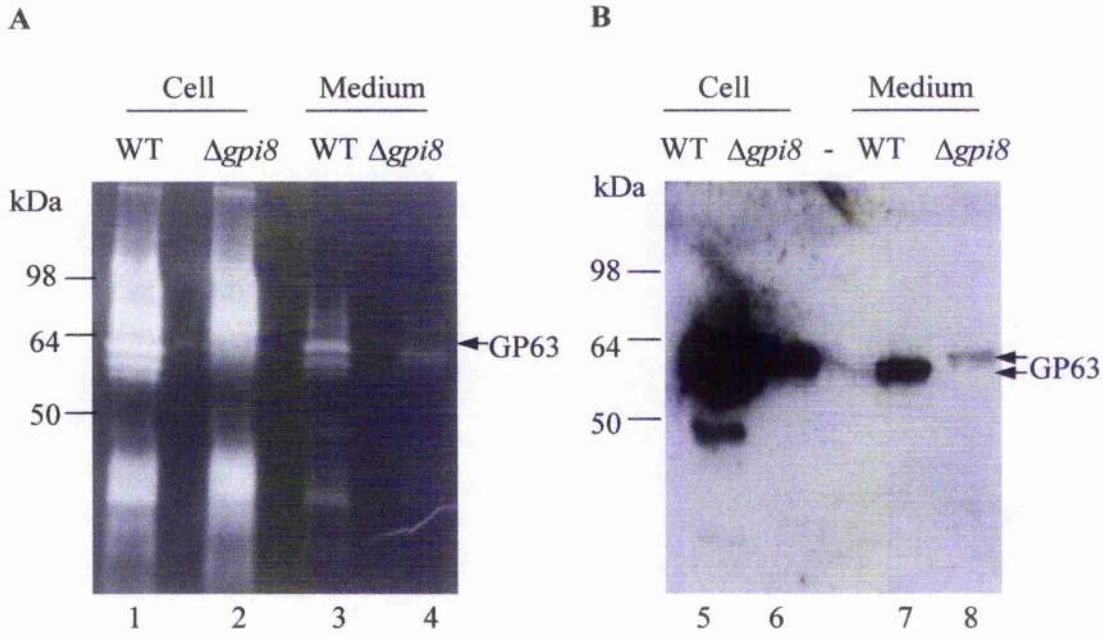


Figure 4.13: Detection and gelatinolytic activity of proteins precipitated with ConA from WT and Δ*gpi8* cells grown in the absence of FCS.

Cells were grown to late log phase in medium containing 5% FCS. $1.7x10^8$ WT cells and $3.4x10^8$ Δ*gpi8* cells were washed and grown in 5mls of HOMEM in the absence of FCS for 6 hours. Medium and cell samples were ConA precipitated.

Panel A) Detection of gelatinolytic activity. $3x10^7$ WT and $6x10^7$ Δ*gpi8* cell equivalents from both medium and cell were ConA precipitated and samples electophoresed on a 10% SDS-PAGE gel containing 0.1% gelatin. The gel was treated for gelatinolytic activity by incubating in 50mM Tris-HCl pH8.00 at 37°C overnight.

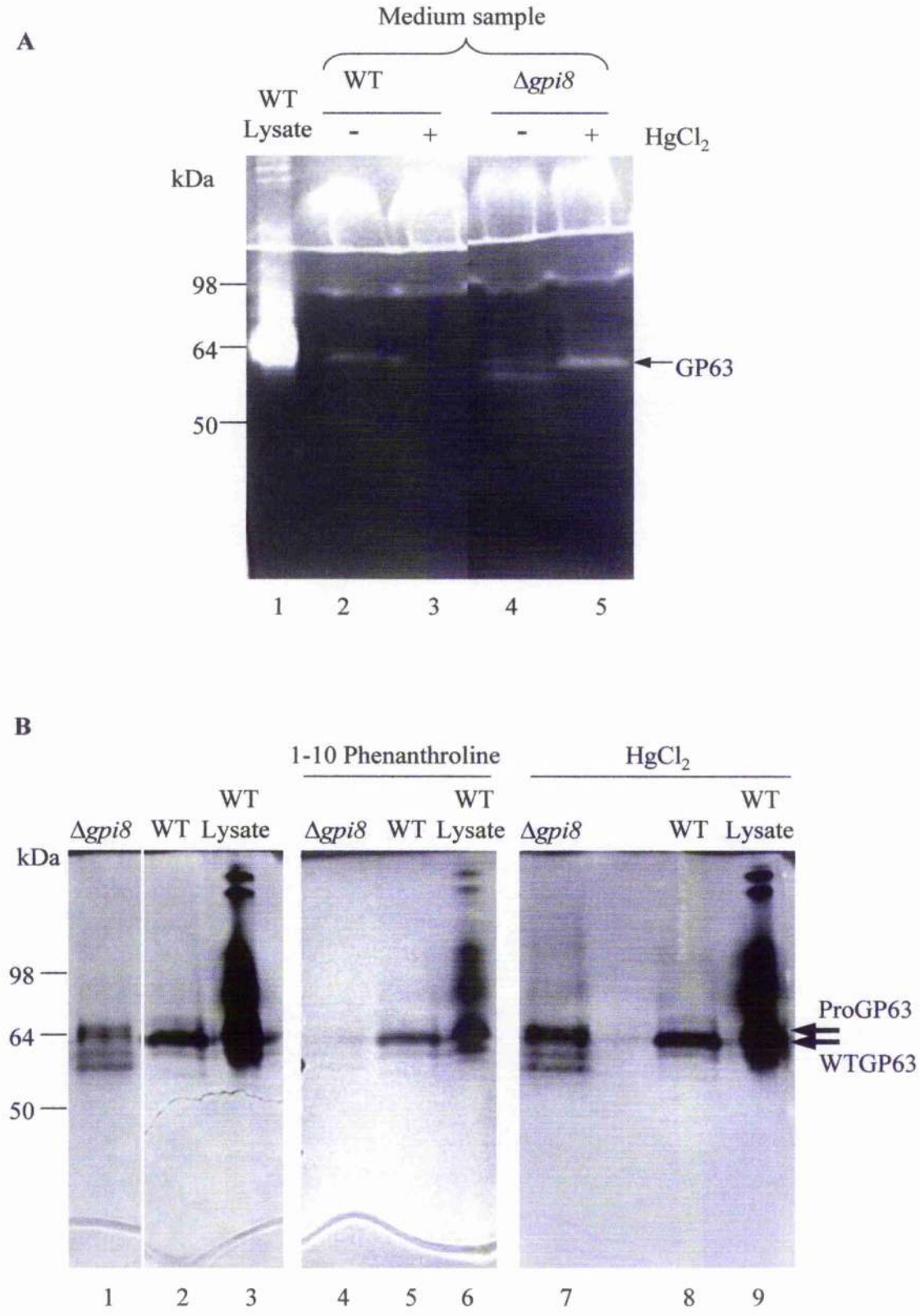
Panel B) Detection of GP63. $3x10^7$ WT and $6x10^7$ Δ*gpi8* cell equivalents from both medium and cells were ConA precipitated, and samples electophoresed by 12% SDS-PAGE, electroblotted and GP63 detected by western blotting.

Figure 4.14: Activation of secreted GP63 with $HgCl_2$.

Panel A) WT and Δ*gpi8* cells were grown to late log phase, washed, and resuspended in medium lacking FCS for 4 hours. The medium samples weres collected, pre-cleared and ConA precipitated. The eluted samples were then treated with or without 2μM $HgCl_2$ at 37°C for 1 hour. WT lysate from $1x10^7$ cell equivalents (Lane 1), and the medium from an estimated $6x10^7$ cell equivalents (Lanes 2-5) were loaded on a 10% SDS PAGE gel containing 0.1% gelatin. Subsequent to electrophoresis the gel was washed in 2.5% TX-100, and treated for gelatinolytic activity by incubation in 50mM Tris HCl pH8.00 at 37°C overnight.

Panel B) WT and Δ*gpi8* cells were grown to late log phase, washed, and resuspended in medium lacking FCS for 4 hours. The medium samples were collected, pre-cleared and ConA precipitated. WT lysate from $5x10^6$ cell equivalents (Lanes 3, 6 and 9), and the medium from an estimated $2x10^8$ cell equivalents (WT lanes 2, 5 and 8, Δ*gpi8* lanes 1, 4, and 7) were loaded on a 10% SDS PAGE gels containing 0.1% gelatin. Subsequent to electrophoresis the gels were washed in 2.5% TX-100, and treated for gelatinolytic activity by incubation in 50mM Tris HCl pH 8.00 at 37°C overnight. In addition one gel (Lanes 4-6) was also incubated with 20mM 1-10 phenanthroline, and a second gel (Lanes 7-9) with 4μM $HgCl_2$, during the final wash and incubation steps. (Note this picture was taken with inverted contrast).

The proteins predicted to be GP63 are indicated .


A
Medium sample
WT
Δgpi8
WT Lysate
-
+
-
+
HgCl2
kDa
98
64
50
GP63
1
2
3
4
5
B
1-10 Phenanthroline
HgCl2
Δgpi8
WT
WT Lysate
kDa
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50
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WTGP63
1
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8
9

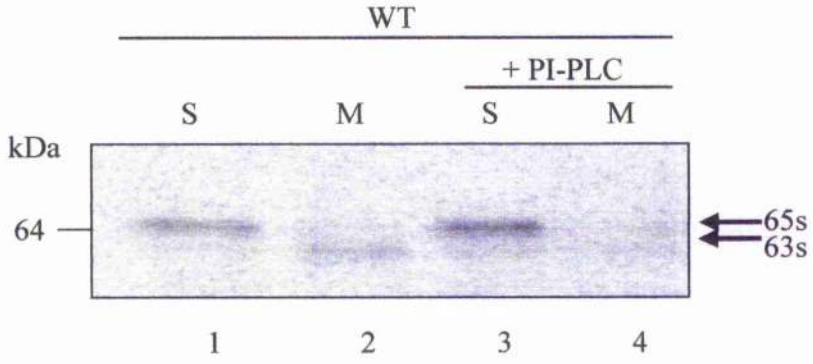


Figure 4.15: Analysis of the secreted forms of GP63.

WT cells were grown to mid log phase and $1x10^8$ cells were metabolically labelled with ^{35}S-ExpreSS for 6 hours. The medium was collected, pre-cleared and the samples split into 2 and treated with or without PI-PLC for 1 hour prior to TX-114 fractionation. Soluble (S), and membrane-associated (M) fractions were and immune-precipitated with a GP63 antibody (L3.8).

Samples were electrophoresed on a 12% SDS-PAGE gel and visualised using a phosphor imager. The 65s and 63s forms of GP63 are indicated.

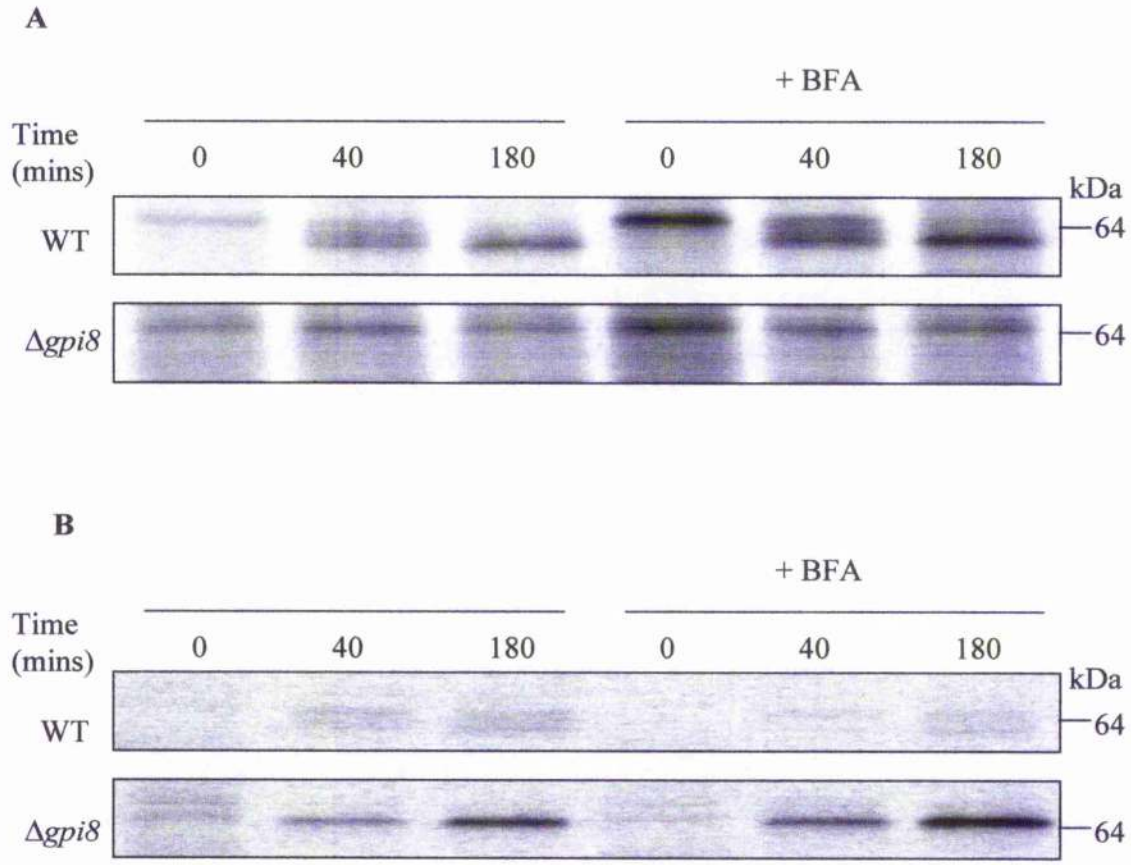


Figure 4.16: Brefeldin A Treatment of cells.

Cells were grown to mid log phase and treated with 10μg ml^{-1} Brefeldin A (BFA) for 4 hours prior to pulse chase labelling. WT and Δ*gpi8* promastigotes were labelled with ^{35}S-ExpreSS for 12 minutes and then chased in cold medium for a period up to 180 minutes. Samples were collected at appropriate time points.

Panel A) Cells were lysed, immune-precipitated with an αGP63 antibody (L3.8), and electrophoresed on a 12% SDS-PAGE gel.

Panel B) Samples of medium were pre-cleared, immune-precipitated with an αGP63 antibody (L3.8), and electrophoresed on a 12% SDS-PAGE gel.

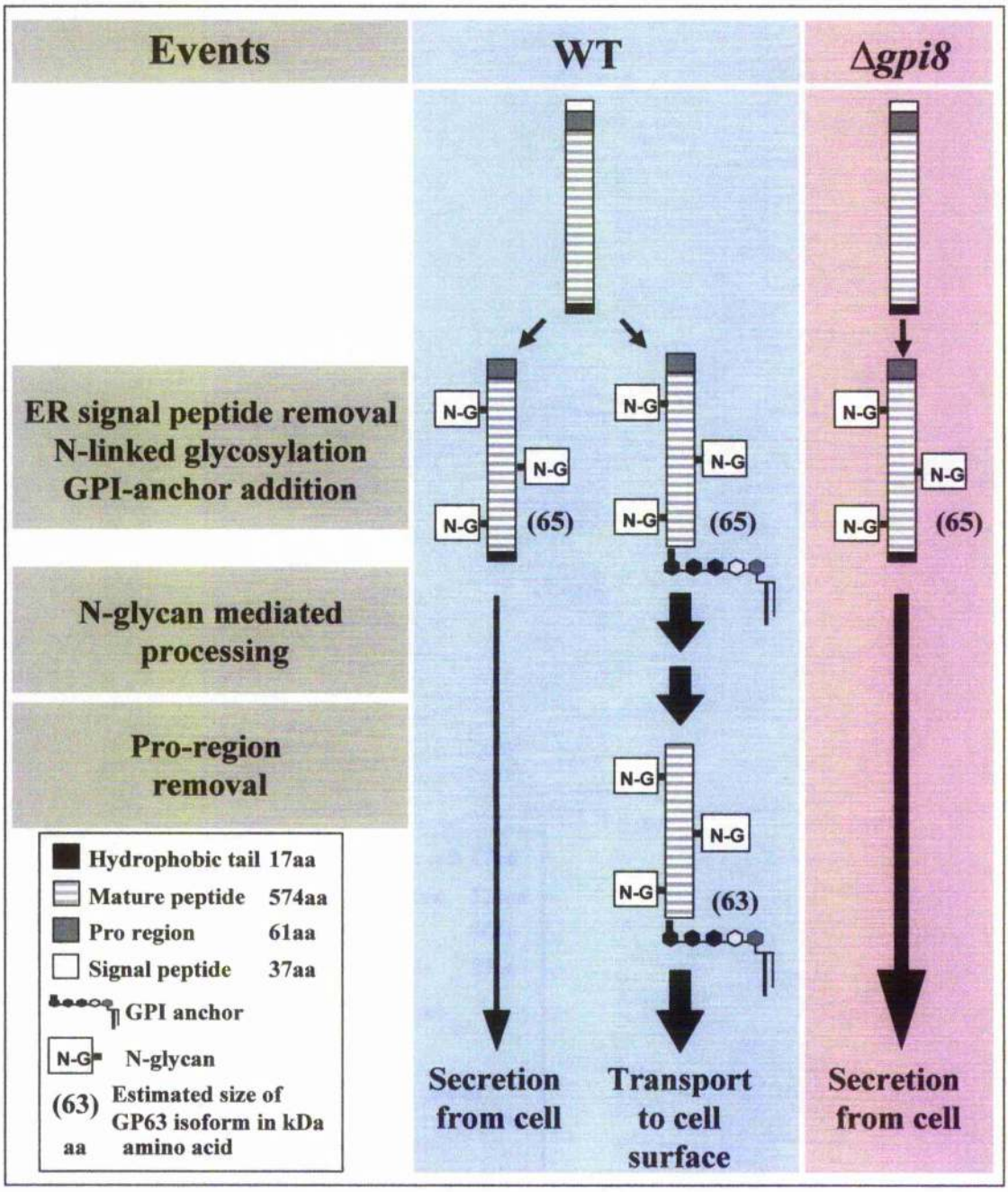


Figure 4.17: Model of GP63 processing in WT and Δ*gpi8* cells.

In WT cells the majority of GP63 is N-glycosylated and GPI-anchored rapidly in the ER to give a 65kDa form. N-glycan processing (not defined in this model) and pro-domain removal give a mature, active 63kDa isoform that is transported to the the cell surface. In WT cells some GP63 is N-glycosylated and secreted from the cell without further modification. In Δ*gpi8* all GP63 destined for secretion is N-glycosylated and transported from the cell without further modification.

Chapter 5

Identification of GPI biosynthesis and trafficking components

5.1 Introduction

GPI anchor biosynthesis has been well characterised in mammalian cell lines (described in section 1.4). However, in *Leishmania* sp. only GPI8, the catalytic subunit of GPIT, has been identified. It seems likely that other members of the GPIT complex exist, mimicking the situation found in yeast and mammalian cells. The production of the Δ*gpi8* cell line demonstrated that GPI anchoring is not essential in *L. mexicana* (Hilley *et al.*, 2000) as it is in other organisms such as yeast (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996), suggesting that *L. mexicana* promastigotes are a suitable model organism in which to study GPI anchor biosynthesis by the production of null mutants. GPI anchors are also speculated to have some involvement in the trafficking of proteins (Muñiz and Riezman, 2000). This chapter describes two approaches taken to identify novel genes involved in the GPIT complex, GPI biosynthesis or the trafficking of GPI-anchored proteins in *L. mexicana.*

5.2 Epitope tagging of GPI8

Detection of GPI8 has previously only been possible from cell lines re-expressing GPI8 from an episome by immune-precipitation of S^{35} labelled proteins with the R492 polyclonal GPI8 antibody. GPI8 was not detected in WT cells by this method, possibly due to the low level of expression of the protein in these cells, as discussed in chapter 3. A previous study produced a GPI8-green fluorescent protein (GFP) fusion protein, expressed from an episome in the Δ*gpi8* cell line (Hilley, 1999). It was suggested that this fusion protein was only partially able to rescue GPIT activity, as western blotting with a GP63 antibody detected only a 50kDa protein within the Δ*gpi8*[pX*GPI8*-GFP] cell lysate, compared to the 63kDa protein present in WT cell lysate. It was suggested that the size of the GFP component of the GPI8-GFP fusion protein may effect the correct functioning of the GPI8 protein, with respect to complex formation, and interaction with other proteins (Hilley, 1999).

It was thought that the production of a tagged GPI8 protein would provide a tool with which to examine the protein, and associated complex members in greater detail. Tagging GPI8 with a small epitope would produce a fully functional and easily detectable form of the protein. This would allow intracellular localisation of GPI8 by immunofluorescence, and co-immune-precipitation of GPI8 with other members of the GPIT complex, and identification of these proteins.

The TY epitope is a short 10 amino acid peptide from the Ty-1 virus-like particle, an immunologically well-characterised protein found in *S. cerevisiae* (Brookman *et al.*, 1995). The TY epitope has been successfully developed in *T. brucei* as a means of tagging proteins (Bastin *et al.*, 1996). The epitope was readily detected by two monoclonal antibodies, BB2 and TYG-5, in immunofluorescence, immune-precipitation and western blotting analysis (Brookman *et al.*, 1995). The antibodies were produced using hybridoma cell lines (Brookman *et al.*, 1995).

5.2.1 Production of TY tagged GPI8

The original intent of this study was the production of a cell line with GPI8-TY integrated into the GPI8 locus, as this would allow the analysis of the cells expressing the protein at WT levels, rather than examining GPI8 in cells over-expressing the protein from an episome. Initially, production of an episomally expressed TY tagged GPI8 was planned. This would allow the assessment of a suitable position within the protein to detect the epitope and maintain a functional GPI8.

Two TY tagged forms of GPI8 were constructed using the pXGPI8 expressing episome (pGL269) (Hilley *et al.*, 2000). The restriction enzyme sites *Not*I and *Nde*I were identified as unique sites within the plasmid, and were present within the GPI8 ORF. A Kyte-Doolittle plot of GPI8 hydrophobicity demonstrated both restriction enzyme sites were located in hydrophilic regions of the encoded protein, and were therefore suitable positions to insert an epitope tag (Figure 5.1, panel A). Neither enzyme site was within the region of the ORF encoding for the active site histidine or cysteine residues, and insertion of an epitope tag at either position was not predicted to affect GPI8 activity. The *Not*I restriction site was present in the GPI8 ORF close to the region which encoded for the cleavage point of the predicted N-terminal signal sequence thought to target GPI8 to the ER (Figure 5.1 Panel A and B).

Two sets of oligonucleotide pairs were designed (Table 5.1). When annealed, each pair produced a small DNA fragment, which encoded for the TY epitope in frame with GPI8, with appropriate overhangs to allow insertion into either the *Not*I or *Nde*I restriction enzyme sites.

Name	Sequence	Restriction enzyme site
TY EPITOPE	E V H T N Q D P L D GAG GTC CAT ACT AAC CAG GAT CCA CTT GAC CTC CAG GTA TCA TTG GTC CTA GGT GAA CTG	
OL532 OL533	5′ 3′ G GCC GAG GTC CAT ACT AAC CAG GAT CCA CTT GAC GC CTC CAG GTA TGA TTG GTC CTA GGT GAA CTG CGC CGG 3′ 5′	*Not*I
OL534 OL535	5′ 3′ T ATG GAG GTC CAT ACT AAC CAG GAT CCA CTT GAC CA AC CTC CAG GTA TGA TTG GTC CTA GGT GAA CTG GTA T 3′ 5′	*Nde*I

Table 5.1: Oligonucleotides used in the production of 2 forms of GPI8 tagged with the TY epitope.

The DNA fragments encoding the TY epitope were prepared by mixing 12μg each of the appropriate oligonucleotides, incubating at 96°C, and then slowly cooling to 55°C to allow annealing. The fragments were ligated into the pGL269 plasmid, which had previously been prepared by digestion with either the *Not*I or *Nde*I restriction enzymes. Colonies containing plasmids with the correctly orientated insert were identified by colony PCR, using the primers OL533 and OL460 for insertion at the *Not*I site, and OL535 and OL459 for insertion at the *Nde*I site, and named pGL452 and pGL453 respectively. Plasmids were sequenced to verify that only a single insertion had occurred at each site, and that the GPI8 ORF remained in frame. The plasmids pGL452 and pGL453 were transfected into the Δ*gpi8* cell line to produce the cell lines Δ*gpi8*[pX*gpi8*-TY^{NotI}], and Δ*gpi8*[pX*gpi8*-TY^{NdeI}].

5.2.2 Analysis of TY tagged GPI8

The cell lines Δ*gpi8*[pX*gpi8*-TY^{NotI}] and Δ*gpi8*[pX*gpi8*-TY^{NdeI}] were assessed for GPI8 activity by examining the effect on the GPI anchoring of GP63. Cell lysate material from 10^7 cell equivalents was electrophoresed by 12% SDS-PAGE, electroblotted and

GP63 detected by western blotting (Figure 5.2, panel A). GP63 was detected in the cell lysate material from the WT and Δ*gpi8*[pX*GPI8*] cell lines, and absent from the Δ*gpi8* cell line as shown previously. GP63 was also present in cell lysate material from the Δ*gpi8*[pX*gpi8*-TY^{NotI}] cell line, but absent from the cell lysate material from the Δ*gpi8*[pX*gpi8*-TY^{NdeI}] cell line. This indicated GPI8-TY^{NotI} was active whilst GPI8-TY^{NdeI} was inactive.

The TY epitope in the GPI8-TY^{NotI} mutant was inserted into the protein close to the predicted point of cleavage of the ER signal (Figure 5.1, panel B). This insertion was designed such that in the translated protein removal of the ER signal was not predicted to be effected, and subsequent to the removal of this signal the TY epitope would be positioned close to the N-terminus of the GPI8 protein. In contrast the GPI8-TY^{NdeI} expressing cell line had the epitope inserted internally within the protein. Though it was not predicted to interfere with the active site residues of GPI8, it is possible that the 10 residue peptide has affected the tertiary structure of the protein, possibly disrupting the formation of the GPIT complex, or binding to the GPI-protein thus preventing transamidase activity. In contrast the *Not*I site within the GPI8 ORF therefore provides an ideal position for tagging the GPI8 protein, as it does not appear to affect the function of the GPIT.

The cell lines Δ*gpi8*[pX*gpi8*-TY^{NotI}], and Δ*gpi8*[pX*gpi8*-TY^{NdeI}] were tested to confirm that the epitope tagged GPI8 was recognisable using the TY antibody BB2 (Brookman *et al.*, 1995). Cell extracts from WT, Δ*gpi8* Δ*gpi8*[pX*GPI8*], Δ*gpi8*[pX*gpi8*-TY^{NotI}], and Δ*gpi8*[pX*gpi8*-TY^{NdeI}] were electrophoresed on a 12% SDS PAGE gel, electroblotted and GPI8-TY detected by western blotting with the BB2 antibody used at a dilution of 1:10 (Figure 5.2, panel B). GPI8 has a predicted size of 42kDa. 2 proteins of 43 and 42kDa appeared as a doublet on the gel, and were detected only in the cell lysates of cell lines expressing the TY tagged form of GPI8 (Lanes 4 and 5). No proteins of this size were detected in the WT, or Δ*gpi8* cells, or in the cell line re-expressing GPI8 from an episome (Lane 2). This suggested that the protein detected was GPI8-TY, and this protein was present as 2 isoforms. Insertion of the TY-Tag at either the *Not*I or *Nde*I sites in the GPI8 ORF resulted in an epitope tagged GPI8 protein recognisable by the BB2 antibody.

Whilst the TY-tagging of GPI8 appeared to be successful the western blot showed that the BB2 antibody also detected an abundant smaller sized protein. This protein was present in all cell lines tested, including the Δ*gpi8* cell line, and so is likely to be a cross-reacting protein. The protein was detected at higher levels than GPI8-TY. In an attempt to prevent the detection of the cross-reacting protein the BB2 antibody was used at higher dilutions. Whilst this resulted in the detection of a lower level of contaminating protein it also resulted in the loss of detection of GPI8-TY. A high stringency wash was introduced in an attempt to prevent the cross-reaction. TBS was used in place of PBS usually used in western blot detection. Western blots were washed in TBS, 0.1% Tween after incubation with the primary antibody. The blot was subsequently washed with 10mM Tris, 0.5M NaCl, 0.5% Tween pH 7.6, and then washed again in TBS, 0.1% Tween. This method had no effect on the level of detection of the contaminating protein. Attempts to further purify the antibody by growth of the hybridoma cell line in Serum Free and Protein Free Hybridoma Medium (Sigma), or purification using a protein G column, failed to either increase the concentration of the antibody or prevent the detection of the cross-reacting protein. The presence of a cross-reacting protein has not been reported in other studies on *T. brucei* (Bastin *et al.*, 1996), and in this study where *T. brucei* cell lysate was used as a control no cross contamination was detected. It was concluded that the BB2 antibody detected a cross–reacting protein at high levels in *L. mexicana* cell lysates.

5.3 Production of GPI-anchored GFP

The identification of novel genes involved in GPI anchor biosynthesis and the trafficking of GPI-anchored proteins, requires the development of a novel method to mutate and screen a large number of genes. Therefore a model system was designed to allow the production and assessment of a large number of mutant cell lines. The general approach was to subject *Leishmania* to mutagenesis, screen for mutants lacking in GPI-anchored surface proteins, and complement these mutants using an *L. mexicana* cosmid library. As *L. mexicana* is a diploid organism, 2 random mutation events are required to produce recessive mutants. The frequency of recovery of null mutants after chemical mutagenesis is estimated to be 10^{-7} (Guciros-Filho and Beverley, 1996). The method used would therefore require the efficient screening of a high number of cells for the loss of GPI-anchored proteins.

The green fluorescent protein (GFP) from *Aequorea victoria* is commonly used as a marker for gene expression in eukaryotic cells (Chalfie *et al.*, 1994). Expression of GFP, enhanced GFP, and a GFP tagged protein from the pXG episome in *L. major* and *L. donavani* demonstrated that the protein was a suitable marker for use in *Leishmania* (Ha *et al.*, 1996). Use of fluorescence activated cell sorting (FACS) demonstrated that separation of GFP expressing cells from non-GFP expressing cells in a mixed population was possible (Ha *et al.*, 1996). It was therefore thought that the construction and use of a GPI-anchored GFP expressing episome in *L. mexicana* would allow the rapid detection of GPI biosynthesis mutants by FACS analysis.

It was predicted that a GP63-GFP fusion protein with both the N-terminal signal peptide and the C-terminal GPI anchor attachment domain from GP63, would be GPI-anchored and then trafficked to the cell surface. Cells with a GPI-anchored GFP could be isolated from cells with no surface expressed GFP by FACS. Subsequent to chemical mutagenesis, cells with a deficiency in any gene involved in the GPI biosynthesis or trafficking pathways, would not correctly process and traffick GPI-GFP to the surface, and could be isolated by FACS. Similarly those cells in which GPI-anchoring was rescued by complementation with the cosmid library, would regain surface anchored GFP, again making them identifiable by FACS. Rescued cell lines would be produced, and the cosmids isolated to allow the identification of the novel genes (See figure 5.3). Use of GFP and FACS analysis would therefore allow the processing of high numbers of live cells at each stage of the analysis.

5.3.1 Production of GPI-GFP constructs

GPI-anchored proteins have two domains essential for GPI anchor addition, the N-terminal ER signal and C-terminal GPI anchor addition site. GP63 also has a characteristic pro-domain that is cleaved during the trafficking of the protein. A recent study examined the intracellular trafficking of *T. brucei* cathepsin L-like cysteine protease in both *T. brucei* and *L. mexicana* (Huete-Pérez *et al.*, 1999). This demonstrated that the pro-domain, required to maintain the cysteine protease in an inactive form, was required to traffic a pro-domain-GFP fusion protein to the lysosome/ endosome. It was shown that this was due to a 9 amino acid motif present in the pro-domain, thought to be required to direct the trafficking of the cathepsin L-like protein

through the golgi (Huete-Pérez *et al.*, 1999). It seemed possible that the GP63 pro-region may be required for the correct trafficking of the GP63 to the surface.

Two separate constructs were designed to produce GPI-GFP fusion proteins. The two proteins varied at the N-terminus. Both fusion proteins had the GP63 ER-signal, however GFP^{GPI} contained only a short section (15 residues) of the GP63 pro-region. The other protein, $ProGFP^{GPI}$, contained the entire GP63 pro-region and a short section (18 residues) of the N-terminal end of the GP63 mature peptide fused to the N-terminus of GFP (Figure 5.4).

A number of previous studies have converted non-GPI-anchored proteins to GPI-anchored molecules by addition of the GPI-attachment site, hydrophilic spacer and hydrophobic domain from GPI-anchored molecules (Micanovic *et al.*, 1990; Moran and Caras, 1991). However in one study the non-GPI-anchored yeast protein prepro-α-factor (ppαf) failed to become GPI-anchored when the ω site, hydrophilic spacer, and hydrophobic domain from the GPI-anchored yeast protein Gas1p was added to ppαf to produce a fusion protein. GPI anchor addition did occur when a second fusion protein incorporating a further 20 amino acids N-terminal of the Gas1p ω site was produced (Doering and Schekman, 1997). The reason for this was unclear, however both GFP-GPI fusion proteins for expression in *L. mexicana* were designed such that the C-terminal end of GFP was fused to an additional 43 residues of the mature peptide of *L. mexicana* GP63, prior to the ω site, hydrophilic spacer and hydrophobic tail.

The 2 GFP-GPI expressing episomes were constructed in several stages (Figure 5.5). Initially the 5' and 3' regions of the GP63 ORF were cloned by PCR from the plasmid pGL454, which contains a cDNA of the C2 type GP63 (GPI-anchored) cloned into pBlueScript (Medina-Acosta *et al.* 1993). The primers used for the cloning of the 5' region of GP63 incorporated a *Sma*I site into the forward primer (OL 763), and an *Eco*RI site into the reverse primers (OL739 and OL741). The primers used for cloning the 3' region of GP63 incorporated a *Hin*dIII site into the forward primer (OL765), and a *Bam*HI site into the reverse primer (OL764).

GFP was cloned by PCR from a commercial vector (pEGFP-C1, Clontech, renamed pGL312) containing enhanced GFP (eGFP). Enhanced GFP is a modified form of the WT protein, which is brighter and has an excitation and emission spectra similar to that of FITC (Cormack *et al.*, 1996). The forward primer (OL740) incorporated an additional *Eco*RI site. The reverse primer (OL613) spanned the region of the multiple cloning site at the 3' end of the GFP ORF.

Purpose	Primer name	Sequence Added restriction enzyme sites are indicated
5'GP63 ORF. Forward	OL763	CCC GGG ATG TCC GTC GAC AGC AGC AGC *Xma*I
5'GP63 ORF Reverse (ER signal)	OL739	GAA TTC CGC GTC GTG GTG GAT GCA GCG GTG *Eco*RI
5' GP63 ORF. Reverse (ER signal and pro-region)	OL741	GAA TTC GGT GAG GTC TTC GGC GGA GAC *Eco*R1
3'GP63 ORF Forward	OL765	GAA GCT TGC ACG CCG GGC CTC AGG TTT *Hin*dIII
3' GP63 Reverse. Downstream of ORF	OL764	CGG ATCC GAC AGC ACC AGT CCT ACC *Bam*HI
GFP ORF. Forward	OL740	GAA TTC GTG AGC AAG GGC GAG GAG CTG *Eco*RI
GFP ORF. Reverse	OL613	CGC GGT ACC GTC GAC TGC

Table 5.2: Primers used in the production of the episomes pGL586, and pGL587, encoding the fusion proteins GFP^{GPI} and $ProGFP^{GPI}$ respectively.

PCR reactions used Pfu polymerase, for increased proof reading ability. Subsequent to PCR the reactions were heated to 96°C for 30 minutes, and then 1U of Taq polymerase, 20μM ATP added and the reactions incubated at 72°C for 2 min. This incorporated an additional adenosine overhang to the PCR products to allow direct cloning into the commercial vector pGEMT (Promega). The plasmids were named pGL540 (GP63 ER signal, and pro-region), pGL541 (GP63 ER signal), pGL542 (GFP ORF), and pGL556 (GP63 C-terminal). Plasmids were checked for insert orientation by restriction digests, and sequenced using the SP6 and T7 primers to check that no mutations had been incorporated by PCR.

The plasmid pGL542 was digested with the enzyme *Eco*RI to release the GFP ORF, and the GFP *Eco*RI fragment was cloned into the *Eco*RI site of both pGL540 and pGL541, to produce plasmids pGL558 and pGL557 respectively. Insertion of the GFP *Eco*RI fragment in the correct orientation was verified by restriction digestion. The plasmid pGL556 was digested with the restriction enzymes *Hin*dIII and *Pst*I to release the 3' fragment of the GP63 ORF, and this fragment was ligated into the plasmids pGL558 and pGL557 at the *Hind*III and *Eco*RI sites, to create plasmids pGL576 and pGL575 respectively.

The final step of the cloning was restriction digestion of the plasmids pGL576 and pGL575 with the enzymes *Sma*I and *Bam*HI and ligation into the *Sma*I and *Bam*HI sites of the pXG episome, to produce the episomes pGL587 and pGL586. The pXG episome is a *Leishmania* expression vector with high expression levels (Ha *et al.*, 1996). The episomes pGL586 and pGL587 were shown, by both restriction digestion and sequencing, to contain no mutations, and the predicted proteins from the sequence correlated with the fusion proteins as originally designed. The episomes pGL586 and pGL587 were transfected into WT *L. mexicana* to generate the cell lines WT[pXGGFP^{GPI}] and WT[pXGProGFP^{GPI}] respectively. The episomes were also transfected into the Δ*gpi8* cell line for comparison generating the cell lines Δ*gpi8*[pXGProGFP^{GPI}] and Δ*gpi8*[pXGGFP^{GPI}]. The episome pXG-GFP+ (renamed pGL104, a gift from Dr Steve Beverley, Washington University Medical School), was transfected into WT cells generating the cell line WT[pXGFP]. The plasmid contains the GFP coding region, inserted into the *Sma*1 site of the pXG episome (Ha *et al.*, 1996).

5.3.2 Analysis of GFP-GPI expressing cell lines

Live cells were examined directly by fluorescence microscopy to assess GFP expression. Cells were grown to mid log phase, washed 3 times and resuspended in PBS. NaN_3 was added to a concentration of 0.005% to prevent excessive movement of the live cells, and cells were examined by fluorescence microscopy on a Zeiss microscope (Figure 5.6).

WT cells showed a low level of background fluorescence (Panel A). In contrast the positive control WT[pXGFP] showed a high level of fluorescence which appeared

cytosolic in its distribution (Panel B), as has been described previously when the plasmid was expressed in *L. major* (Ha *et al.*, 1996). A mixed population of cells was present with regards to the level of GFP expression (Compare arrowed cells in Panel B), and in some cases individual cells did not appear to express GFP at all. All cultures were grown in the presence of G418, therefore the episome would be expected to be present in all cells.

WT cells expressing the GFP^{GPI} fusion protein showed a different distribution of fluorescence compared to those expressing GFP (Panel C). The protein was not located on the cell surface, but instead remained intracellularly, in an extranuclear pattern. The protein was not distributed evenly throughout the cytoplasm but instead appeared in a distinct pattern consistent with an ER location. A similar location was also found in the $ProGFP^{GPI}$ expressing cell line (Panel D). Analysis of the Δ*gpi8*[pXG$ProGFP^{GPI}$] cell line showed a showed a high level of fluorescence, with a similar pattern of distribution (Panel F). The Δ*gpi8*[pXGGFP^{GPI}] cell line did not show fluorescence at a level comparable with the other GFP expressing cell lines (Panel E). The intensity was at a level similar to WT cells and was therefore indistinguishable from background fluorescence. GFP expression could not be detected in this cell line by fluorescence microscopy. The three cell lines expressing detectable GFP^{GPI} or $ProGFP^{GPI}$ also showed high variability in the level of fluorescence detectable in individual cells (See cells indicated by arrows in figure 5.6).

The four cell lines were examined for GFP expression by western blotting. Cells were grown to mid log phase, and 10^7 cell equivalents were electrophoresed by 12% SDS-PAGE, electroblotted, and GFP detected using a mouse monoclonal GFP antibody (Clonetech-JL8) at a dilution of 1:1000 (Figure 5.7). No protein was detected in either the WT or Δ*gpi8* cell lysate material (Lanes 1 and 2), whilst a protein of approximately 72kDa was detected in cell lysate material from the WT[pX*GPI8*-GFP] cell line (Lane 7). This was the estimated size of the GPI8-GFP fusion protein and suggested that the antibody was detecting GFP. Protein was detected in the cell lysate material from the WT[pXGGFP^{GPI}], WT[pXG$ProGFP^{GPI}$] and Δ*gpi8*[pXG$ProGFP^{GPI}$] cell lines (Lanes 4-6), suggesting that these cell lines were expressing a form of GFP. The predicted size of GFP^{GPI} was approximately 35-42kDa, dependent on whether processing events such as

the removal of the ER signal and or the GPI attachment signal occurred. The estimated size of ProGFP^{GPI} was 49-35kDa, dependent on the removal of the ER signal, pro-region and GPI-attachment signal. Whilst proteins within this range were present in each of the cell lines, it was not clear which of the detected proteins represented the GFP fusion proteins. It would be anticipated that the ProGFP^{GPI} protein would be of a larger size than GFP^{GPI} unless pro-region removal had occurred. Removal of the pro-region of GP63 occurs by an unknown mechanism during the trafficking of GP63, possibly by enzymes localised in a membrane trafficking pathway (Macdonald *et al.*, 1995). In the absence of the trafficking of the GFP fusion proteins to the cell surface, it might be predicted that this process would not occur. However there was no difference in the size of the detected protein in the cell lysates expressing the 2 different fusion proteins (compare lanes 5 and 6). No protein of the predicted size, 35-42kDa, was detected in the Δ*gpi8*[pXGGFP^{GPI}] cell line (Lane 3). This result was consistent with the results from the fluorescence microscopy, where GFP expression was not detected. This suggested that the fusion protein GFP^{GPI} was not expressed in these cells, and the episomal expression of GFP^{GPI} was lost. It was also possible that the GFP^{GPI} protein was degraded or rapidly secreted from the Δ*gpi8*[pXGGFP^{GPI}]cell line. A protein of size 26kDa was present in each of the 5 cell lysates expressing GFP fusion proteins (Lanes 3 to 7). It is possible that the multiple proteins detected represent different isoforms or degradation products of ProGFP^{GPI} and GFP^{GPI}, the fusion proteins having undergone a variety of processing or degradation events within the cell.

Over a period of weeks the WT and Δ*gpi8* transfected cell lines lost GFP^{GPI} or ProGFP^{GPI} expression, as determined by western blotting and fluorescence microscopy. Growth of the original stabilates in increasing concentrations of the antibiotic G418 failed to prevent this loss of expression. Expression of GFP^{GPI} and ProGFP^{GPI} from an episome appeared to be unstable.

The results from the fluorescence microscopy indicated that the GFP-GPI fusion proteins failed to reach the surface of WT cells. The pattern of intracellular fluorescence suggested that the proteins were targeted to the ER. However it was not clear if the proteins received a GPI anchor, and failed to be trafficked from the ER or if

the proteins did not receive an anchor. TX-114 fractionation and PI-PLC treatment were used in an attempt to address this question.

Cells were grown to mid log phase and 10^8 cells were washed in PBS, pelleted, and resuspended in 300µl of TX-114 buffer but in the absence of TX-114, and the addition of 0.05% TX-100 and incubated at room temperature for 30 minutes to lyse the cells. Samples were pre-cleared by centrifugation and the supernatant transferred to a fresh tube. Each sample was divided in two and 2µl of PI-PLC was added to one of each pair. All samples were incubated at 37°C for 1 hour, and pre-condensed TX-114 added to a final concentration of 0.5%. All samples were TX-114 fractionated as described previously (Section 2.5.8). Subsequent to fractionation 10µl of 4 x SDS loading buffer was added to the 30µl of membrane fraction. The soluble fraction was concentrated to 60µl using a Microcon spin column (Amicon), and 20µl of SDS loading buffer added. Samples were boiled and comparable amounts of membrane and soluble fractions were electrophoresed on 12% SDS-PAGE gels. Gels were electroblotted, and then western blotted using either a GP63 monoclonal antibody at a dilution of 1:50 (Figure 5.8, panel A), or the Clontech JL-8 GFP antibody at a dilution of 1:1000 (Panel B, and C).

Western blotting of the WT[pXGGFP^{GPI}] and WT[pXGProGFP^{GPI}] cell lines with a GP63 antibody demonstrated that the PI-PLC treatment and TX-114 fractionation worked efficiently (Figure 5.8, panel A). GP63 was detected in both cell lines, and the protein was detected in the membrane fraction with no PI-PLC treatment, and was present in the soluble fraction subsequent to treatment.

Western blotting using the JL8 GFP antibody (Panel B and C) failed to detect any protein using the SuperSignal west pico chemiluminescent detection kit (Pierce). However using the SuperSignal west femto maximum sensitivity detection kit (Pierce), which has increased sensitivity, did detect some protein. A similar pattern of proteins was present in each of the 4 cell lines. A protein of approximately 63 kDa was detected in all fractions of each cell line. The predicted size of GFP^{GPI} was 35-42kDa, and the predicted size of ProGFP^{GPI} was 49-35kDa, the size of the 63 kDA protein suggested it was a cross-reacting protein. A repeat of the experiment using WT cells would confirm this. A protein of an estimated 30kDa was detected in the soluble fraction of each cell

line, and proteins within the predicted size range of ProGFP^{GPI} were detected in the Δ*gpi8*[pXGProGFP^{GPI}], minus PI-PLC, soluble cell fractions. However, no protein was detected which was judged to be GPI-anchored, as assessed by partitioning into the detergent fraction, and movement to the soluble fraction subsequent to PI-PLC treatment. This suggested that the GFP fusion proteins were not GPI-anchored. However the technical problems in detecting the GPI-GFP, made this experiment inconclusive. The difficulty in detecting the GFP fusion proteins by this method, may have been due to the degradation of the proteins within the cells, though this prediction is inconsistent with the GFP fluorescence seen in live cells by microscopy. Alternatively the JL8 antibody may unsuitable for detecting GFP proteins by western blotting. A more sensitive method may be the use of the antibody in immune-precipitation experiments, as GPI8-GFP has previously been detected by this method (see figure 3.2).

5.4 Discussion

The TY tagging of GPI8 was successful, however, a cross-reacting protein was detected at high levels in *L. mexicana* cell lysates using the BB2 antibody. This cross-reaction was not detected in previous work on TY-tagged proteins expressed in *T. brucei* (Brookman *et al.*, 1995), or *Leishmania* (K. Gull, personal communication). The low level of WT GPI8 expression and the presence of the cross-reacting protein prevented the use of the TY-tagged protein for the co-immune-precipitation of other GPIT-complex members from *L. mexicana* using the BB2 antibody. A second TY antibody, TYG-5, has been produced (Brookman *et al.*, 1995). This antibody may not detect the same cross-reacting protein in *L. mexicana* cell lysates, and would therefore make a viable alternative to the BB2 antibody. The TY-tagging of GPI8 has allowed the identification of GPI8 by western blotting, which was previously not possible. This suggested two different isoforms of GPI8 were present within the cell lysate. The *Not*I restriction site has also been identified as a suitable position for tagging GPI8 without inactivating the protein. GPI8-TY^{NotI} was shown to be active, as GP63 was present in the cell lysate material of the Δ*gpi8*[pX*gpi8*-TY^{NotI}] cell line. The tagging of GPI8 with alternative epitopes inserted at the *Not*I site would provide a method to allow the further analysis of *L. mexicana* GPI8 in the future. A recent study analysed the cross-reactivity of commercially available antibodies to commonly used epitope tags with

Leishmania cell lysates (Traub-Cscko *et al.*, 1998). This demonstrated that anti-c-myc cross-reacted with a 17kDa protein, and anti-FLAG with a 36kDa protein in *L. mexicana* lysates. A suitable alternate tag might be the HA epitope, a 9 amino acid peptide present on the human influenza virus hemaglutinin protein (Wilson *et al.*, 1984). The HA antibody has been used to successfully detect a HA-tagged form of dolichol-phosphate-mannose synthase (DPMS) in *L. mexicana* (Mullin *et al.*, 2001). GPI8-TY^{Ndel} was an inactive protein. It might be interesting to express this protein in WT cells to examine any possible dominant negative effect, and observe if this differed from that seen when the active site mutant GPI8^{C216G} was expressed in WT cells.

Neither of the fusion proteins GFP^{GPI} or ProGFP^{GPI} became GPI-anchored on the cell surface as anticipated. Fluorescence microscopy indicated that the fusion proteins remained intracellularly with a pattern of fluorescence indicative of an ER location. This distribution was similar to that seen when a GFP construct was expressed with an N-terminal signal sequence and a *T. brucei* ER retention signal in *L. mexicana* (Ilgoutz *et al.*, 1999a). Expression of a GFP-tagged form of the *Leishmania* LPG3 protein in *L. donovani* also showed a similar pattern of fluorescence (Descoteaux *et al.*, 2002). LPG3 is a homologue of the mammalian ER chaperone GRP94. LPG3-GFP co-localised with BiP, a known ER protein, confirming the protein's ER location. Co-localisation studies with known ER proteins, such as the *Leishmania* ER marker LM39 (Wallis *et al.*, 1994) or the molecular chaperone BiP (Bangs *et al.*, 1993) would confirm the ER location of the GFP fusion proteins used in this study. TX-114 fractionation experiments also suggested that the proteins failed to receive a GPI anchor, as all detected proteins which might be ProGFP^{GPI}, or GFP^{GPI}, were detected only in the soluble fraction. The pattern of fluorescence was similar in both WT and Δ*gpi8* cells expressing the constructs, suggesting that the presence or absence of a functional GPIT complex had no effect on the intracellular distribution of the fusion proteins within the cells.

The reason for the failure to produce GPI-anchored GFP using this system is unclear. GPI-anchored GFP fusion proteins have been successfully used in mammalian systems. A GPI-anchored GFP incorporating the ER signal (residues 1-25), and the N-terminal domain (residues 67-102, with the ω site at residue 77) of the GPI-anchored receptor

protein CD59, was shown to be anchored to the surface of CHO cells and the mouse T cell line, EL4 (Hiscox *et al.*, 2002). A fusion protein described as a 'minimal GPI-GFP fusion protein' was successfully expressed on the plasma membrane of a variety of mammalian cell lines (Cos, HeLa, NRK and MDKC) (Nichols *et al.*, 2001). The GPI-GFP behaved in an identical manner to the endogenous mammalian GPI-anchored protein CD59, though a second endogenous GPI-anchored protein, the folate receptor, had a different distribution suggesting that the trafficking of some GPI-anchored proteins may be mediated by additional sorting signals (Nichols *et al.*, 2001). The transferrin receptor (TfR) of *T. brucei* is GPI-anchored. It is expressed in bloodstream form cells and is localised to the flagellar pocket. The different surface location of this protein, compared to VSG, suggests that some sorting or retention mechanism exists to specifically retain TfR within the flagellar pocket (Mußmann *et al.*, 2003). Analysis of the intracellular trafficking of VSG by studying the distribution of the protein within *T. brucei* suggested that the enrichment or sorting of the GPI-anchored protein occurs in several intracellular compartments (Grunfelder *et al.*, 2002). Therefore a variety of signals may be required to correctly target GPI-anchored proteins to the cell surface.

The expression of GFP and GFP fusion proteins has also been used in successfully in *Leishmania*. Similar to the findings in this study, the expression of GFP in *L. major* and *L. mexicana* has previously been shown to result in a cytoplasmic distribution (Ha *et al.*, 1996; Huete-Pérez *et al.*, 1999). The construction of different GFP fusion proteins has also allowed GFP to be targeted to different intracellular organelles. A fusion protein incorporating the *T. brucei* cathepsin L like pro-domain with GFP was successfully used to examine the function of the pro-domain in the trafficking of cysteine proteases (Huete-Pérez *et al.*, 1999). A GFP-DPMS (GFP linked dolichol-phosphate mannose synthase) construct has been used as a marker for DPM linked GPI biosynthesis reactions in *L. mexicana*. GFP-DPMS was demonstrated to localise to a multivesicular tubule (MVT), initially thought to be a sub-domain of the ER, but now regarded as an early lysosome (Ilgoutz *et al.*, 1999a; Mullin *et al.*, 2001).

The failure to produce GPI-anchored forms of either GFP, or the mammalian protein PLAP in *T. brucei* was speculated to be due to the low levels of expression and rapid degradation of alien proteins in this system (Bohme and Cross, 2002). The levels of

expression of the fusion proteins seen in the present study appeared initially high, as the proteins were readily detectable by microscopy. However this level of expression appeared to be unstable and was lost over a period of weeks, even under continued drug selection to maintain the presence of the episome. In contrast, the cell line expressing GFP from an episome maintained GFP in a cytosolic location. The loss of expression of GFP^{GPI} and $ProGFP^{GPI}$ may have been a result of the retention of these proteins intracellularly within the ER, where the build up of a large quantity of alien protein may have been detrimental to the cells. There would be selection for cells not expressing this protein.

A recent study produced a GPI-anchored GFP expressed in *L. major* promastigotes (Ghedin *et al.*, 2001). An episome was constructed containing GFP flanked by an N-terminal signal peptide, from the *Leishmania donovani* surface enzyme 3'nucleotidase/nuclease (*Ld*3'NT/NU), and the C-terminal GPI signal from *L. chagasi* GP63. This protein was termed $3'SP::GFP::GPI^{GP63}$. The GFP used was amplified from the eGFP plasmid from Clontech. Confocal microscopy suggested that this protein was expressed on the cell surface of *L. major* promastigotes, and was present in the flagellar pocket. Increased episomal expression resulted in the chimera accumulating in a small intracellular compartment (Ghedin *et al.*, 2001).

It is not clear why the $3'SP::GFP::GPI^{GP63}$ chimera would become successfully GPI-anchored (Ghedin *et al.*, 2001) whilst the GFP^{GPI} and $ProGFP^{GPI}$ fusion proteins produced in this study failed to do so. Both studies utilised the C-terminal end of GPI-anchored forms of GP63. Though in this study the final 69 amino acids of the C-terminal end of the protein were used, while in the previous study only the final 30 amino acid were used. This study also incorporated the GP63 signal peptide and pro-region, whilst the earlier study utilised the signal peptide from a transmembrane protein. It is possible that GP63 pro-region on the $ProGFP^{GPI}$ protein acted as an ER retention signal, although this seems unlikely as both $ProGFP^{GPI}$ and GFP^{GPI} were retained in the ER, suggesting that the pro-region was not the cause of ER retention.

Previous studies have demonstrated that the requirements for GPI anchor addition are an N-terminal signal to target the protein to the ER lumen (Caras and Weddell, 1989), a C-terminal hydrophobic domain (Caras *et al.*, 1989), spacer region and a domain of

small amino acids around the ω site (Gerber *et al.*, 1992; Nuoffer *et al.*, 1993; Kodukula *et al.*, 1995). These requirements are discussed fully in section 1.4.8. The constructed fusion proteins had each of these elements, and it was predicted that these proteins would become GPI-anchored in *L. mexicana*. The putative ER location of the protein indicated the N-terminal signal had successfully targeted the fusion proteins to the ER. Therefore it seems that the failure in GPI-anchor addition occurred within the ER. A previous study suggested that there may be different requirements at the anchor addition site between species (Moran and Caras, 1994), however the constructs were based on GP63, a protein native to *L. mexicana*. There appears to be no obvious reason why anchor addition should not occur. A recent study has suggested that there is some variability in anchor addition requirements between individual proteins (Aceto *et al.*, 1999). The comparison of the C-terminus of two individual GPI-anchored proteins when fused to the same mature domain, indicated that individual proteins may have unique requirements for anchor addition (Aceto *et al.*, 1999). It is possible that an additional signal exists in GP63 that is required for GPI anchor addition in *L. mexicana*, and this was not incorporated into the GFP-GPI fusion constructs. In mammalian cells proteins destined to be GPI-anchored directly associate with GPI8 during anchor addition (Spurway *et al.*, 2001; Vidugiriene *et al.*, 2001), and photo cross-linking studies indicate the proteins may also associate with GAA1 another member of the GPIT complex (Vidugiriene *et al.*, 2001). The function of this association is not clear, however it is possible that the GFP constructs were not recognised by other, as yet unidentified components of the GPIT complex in *L. mexicana*, either due to misfolding or because the appropriate binding sites were not incorporated into the constructs. This lack of association may have prevented GPI-anchor addition.

It is interesting that failure to produce a GPI-anchored protein did not result in the secretion of the GFP-fusion proteins from the cell, as was found when GP63 failed to become GPI-anchored in the Δ*gpi8* cell line. Instead the proteins appeared to be retained in the ER. This may be due to the lack of appropriate signals on the protein, or may be that the protein was identified to be misfolded. In higher eukaryotes misfolded glycoproteins are retained in the ER by the calnexin calreticulin system, and subsequently targeted for proteasome degradation (Parodi, 2000; Ellgaard and Helenius, 2001). Whilst little is known about the secretory pathway in trypanosomatids,

calreticulin homologues have been identified in *L. donovani* (Joshi *et al.*, 1996) and *T. cruzi* (Labriola *et al.*, 1999), suggesting a similar system exists in these cells. It is possible that GFP^{GPI} and $ProGFP^{GPI}$ are retained in the ER by this system.

The GPI-anchored GFP fusion proteins were unsuitable for their intended use as a method of screening for GPI biosynthesis or trafficking mutants. However production and manipulation of further GFP-GP63 fusion constructs may provide a means to study the trafficking of GPI-anchored proteins in *Leishmania*, and identify the domains required to allow anchor addition and exit from the ER. In *T. brucei* RNA interference (RNAi) has been successfully used to be produce functional 'knockdowns' of specific proteins (Ngô *et al.*, 1998). More recently the introduction of a RNAi genomic library into *T. brucei* was used to identify genes affecting EP-procyclin expression or modification (Morris *et al.*, 2002). However a similar approach may not be possible in *Leishmania*, as the parasite appears to lack the RNAi pathway (Beverley, 2003). A recent study has developed a *L. mexicana* cell line with a modified surface coat suitable for screening for GPI and GIPL biosynthesis mutants (Naderer and McConville, 2002). The production of the cell line, Δ*lpg2*[*pX NEO GPIPLC*], which lacked both LPG and GPI-anchored proteins from the cell surface, allowed the selection of biosynthesis mutants by growth on ConA. ConA is toxic to the cells as it binds to the mannose residues present in GIPLs and GPI anchors, causing the agglutination of the cells. Selection of GIPL and GPI anchor mutants by this method was not previously possible as the LPG coat masks the GIPL, and GPI anchors. Using this screening method a mutant cell line, DIG1, was identified. Analysis of the GPI and GIPL intermediates established that these cells were deficient at the point of α1-6mannose addition to the common GPI and GIPL core (Naderer and McConville, 2002).

Figure 5.1: Epitope tagging of the GPI8 protein

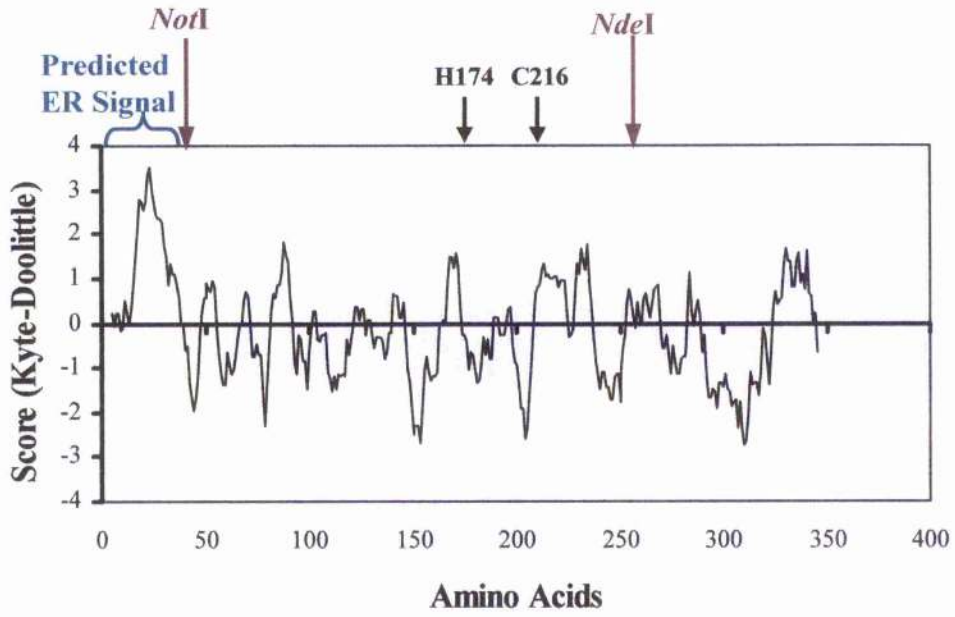
Panel A) Hydrophobicity plot of *L. mexicana* GPI8

Hydrophobicity plot of GPI8 using the Kyte-Doolittle algorithim. Scores greater than zero are hydrophobic, scores less than zero are hydrophilic. The predicted endoplasmic reticulum signal direction sequence from residues 1-31, and the positions of the active site residues H174 and C216 are indicated. The position on the corresponding DNA sequnece of the restriction enzyme sites *Not*I (Amino acid residues 32/33) and *Nde*I (Amino acid residues 256/257) are shown.

Panel B) Predicted amino acid sequence of TY tagged forms of GPI8

The predicted amino acid sequence of the epitope tagged proteins GPI8-TY^{NotI} and GPI8-TY^{NdeI} are shown. The TY epitiope is shaded blue and active site residues shaded pink. An arrow indicates the predicted cleavage point of the ER signal sequence.

A



B

GPI8-TY*NotI*

```
  1  MRTTAYVMTS PTRCIATALI VFAFLVLTAA AEVHTNQDPL DAAAASAPLG
 51  ATGKGQSNNW AVIVSSSRYL FNYRHTANAL TMYHLLRQHG IDDDHILLFL
101  SDSFACDPRN VYPAEIFSQP PGAHDADGRA SMNLYGCSAQ VDYAGSDVDV
151  RRFLSVLQGR YDENTPPTRR LLSDNTSNII IYVAGHGAKS YFKFQDTEFL
201  SSSDISETLT MMHQQRRYGR VVFLADTCHA IALCEHVEAP NVVCLAASDA
251  ESESYSCQYD EQLGTHMVSF WMNEMYLLLN GTSCSNPLTR RIGDDAVSVL
301  HQSWYNFNYH PYRVEASRNR SKPAHRDAVN DPTALREWIV ADFVCGQVSA
351  AVPVDVRYDL E
```

GPI8-TY*NdeI*

```
  1  MRTTAYVMTS PTRCIATALI VFAFLVLTAA AAASAPLGAT GKGQSNNWAV
 51  IVSSSRYLFN YRHTANALTM YHLLRQHGID DDHILLFLSD SFACDPRNVY
101  PAEIFSQPPG AHDADGRASM NLYGCSAQVD YAGSDVDVRR FLSVLQGRYD
151  ENTPPTRRLL SDNTSNIIIY VAGHGAKSYF KFQDTEFLSS SDISETLTMM
201  HQQRRYGRVV FLADTCHAIA LCEHVEAPNV VCLAASDAES ESYSCQYDEQ
251  LGTHMEVRTN QDPLDHMVSF WMNEMYLLLN GTSCSNPLTR RIGDDAVSVL
301  HQSWYNFNYH PYRVEASRNR SKPAHRDAVN DPTALREWIV ADFVCGQVSA
351  AVPVDVRYDL E
```

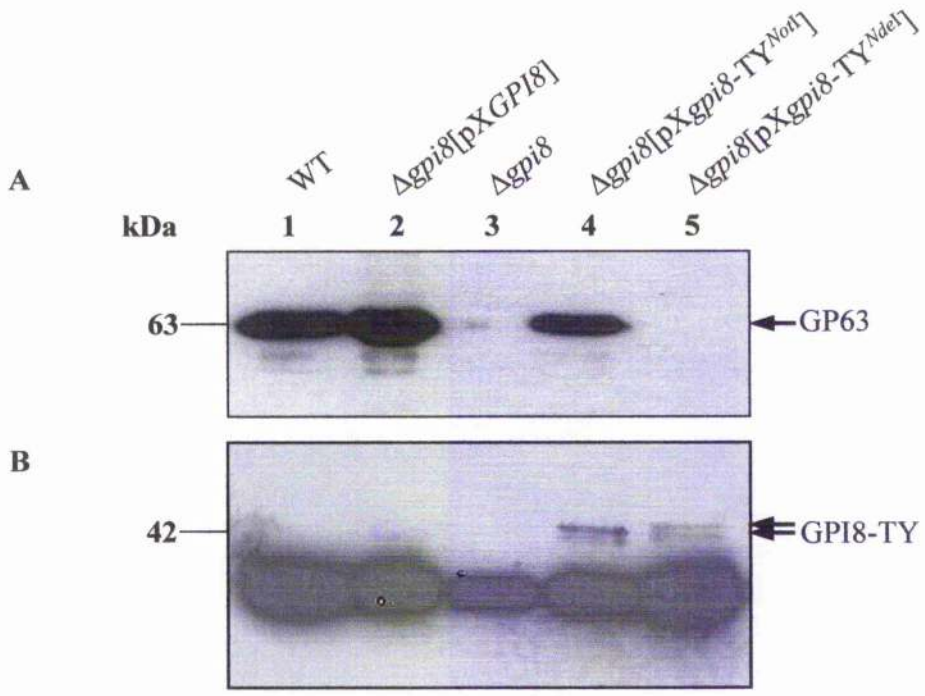


Figure 5.2: Analysis of epitope tagged forms of GPI8

Panel A) Analysis of GP63 expression in cell lines expressing TY tagged forms of GPI8.

Cell lysates were prepared from the cell lines WT, Δ*gpi8*[pX*GPI8*], Δ*gpi8*, Δ*gpi8*[pX*gpi8*-TY^{NotI}], and Δ*gpi8*[pX*gpi8*-TY^{NdeI}]. 1x10^7 cell equivalents per lane were electrophoresed by 12% SDS-PAGE, electroblotted, and exposed to a *L. major* GP63 monoclonal antibody at a dilution of 1:50. The GP63 protein is indicated.

Panel B) Analysis of GPI8-TY expression by western blotting with the BB2 antibody.

Cell lysates were prepared from the cell lines WT, Δ*gpi8*[pX*GPI8*], Δ*gpi8*, Δ*gpi8*[pX*gpi8*-TY^{NotI}], and Δ*gpi8*[pX*gpi8*-TY^{NdeI}]. 1x10^7 cell equivalents per lane were electrophoresed by 12% SDS-PAGE and electroblotted. GPI8-Ty was detected by exposure to the BB2 monoclonal antibody at a dilution of 1:10. The GPI8-TY protein is indicated.

Production of an episomal copy of a GPI-GFP, and transfection of WT *L. mexicana*. Selection of transfectants with G418.

Identification of transfectants by surface fluorescence of live cells

Chemical mutagenesis of transfected cells.

High throughput screening of mutants by FACS analysis. Identification of GPI biosynthesis or trafficking mutants, by isolation of those cells no longer expressing GPI-anchored GFP on the cell surface.

Confirmation that cells lack other GPI anchored-proteins, such as GP63

Gene complementation of the mutant cells using a cosmid library.

High throughput screening of mutants by FACS analysis. Identifiction of complemented mutants, by isolation of those cells re-expressing GPI-GFP on the cell surface.

Isolation of novel genes from the complemented cell lines.

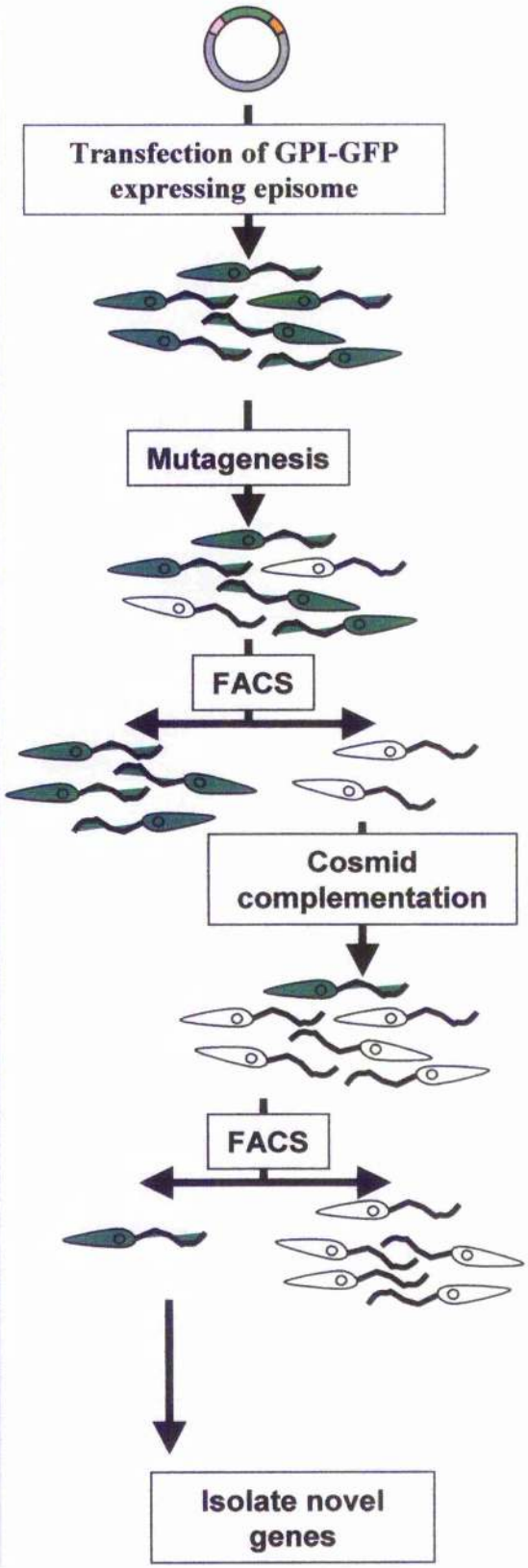
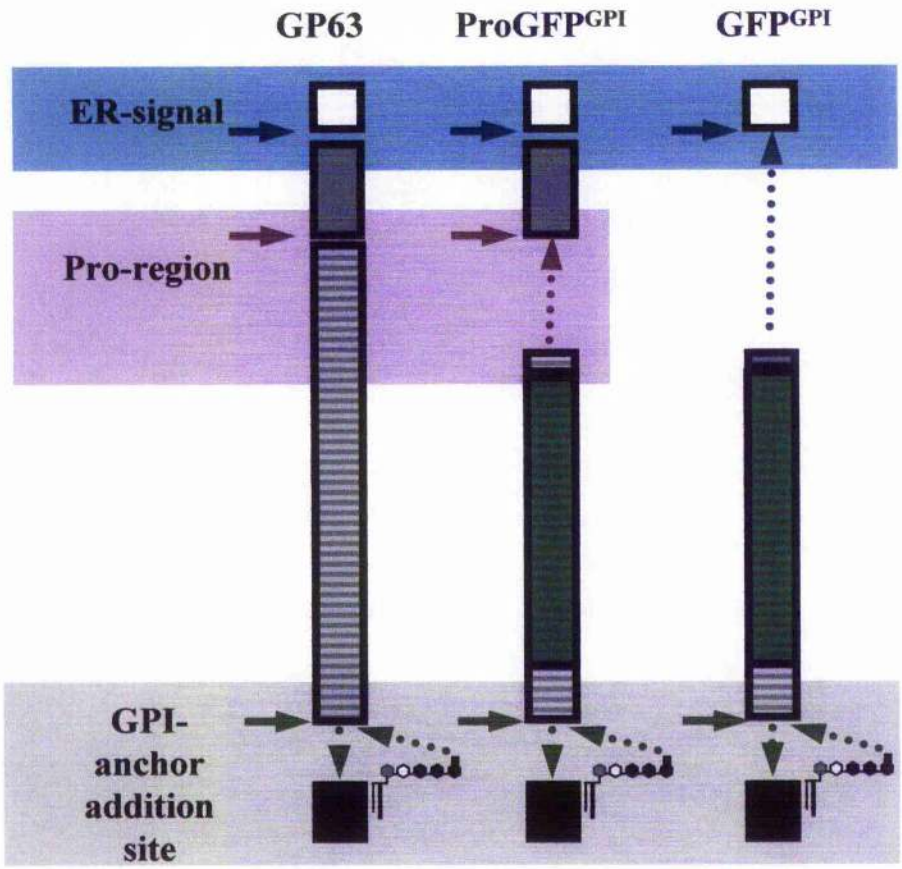


Figure 5.3: Schematic of the method designed for the identification of GPI biosynethesis and trafficking genes in *L. mexicana*



Key	GP63	ProGFP^{GPI}	GFP^{GPI}
ER-signal	37	37	37
Pro-region	63	63	15
Mature GP63	477	19	-
GFP	-	248	248
ω site to GFP	-	44	44
C-terminal end	25	25	25
Total	602	436	369

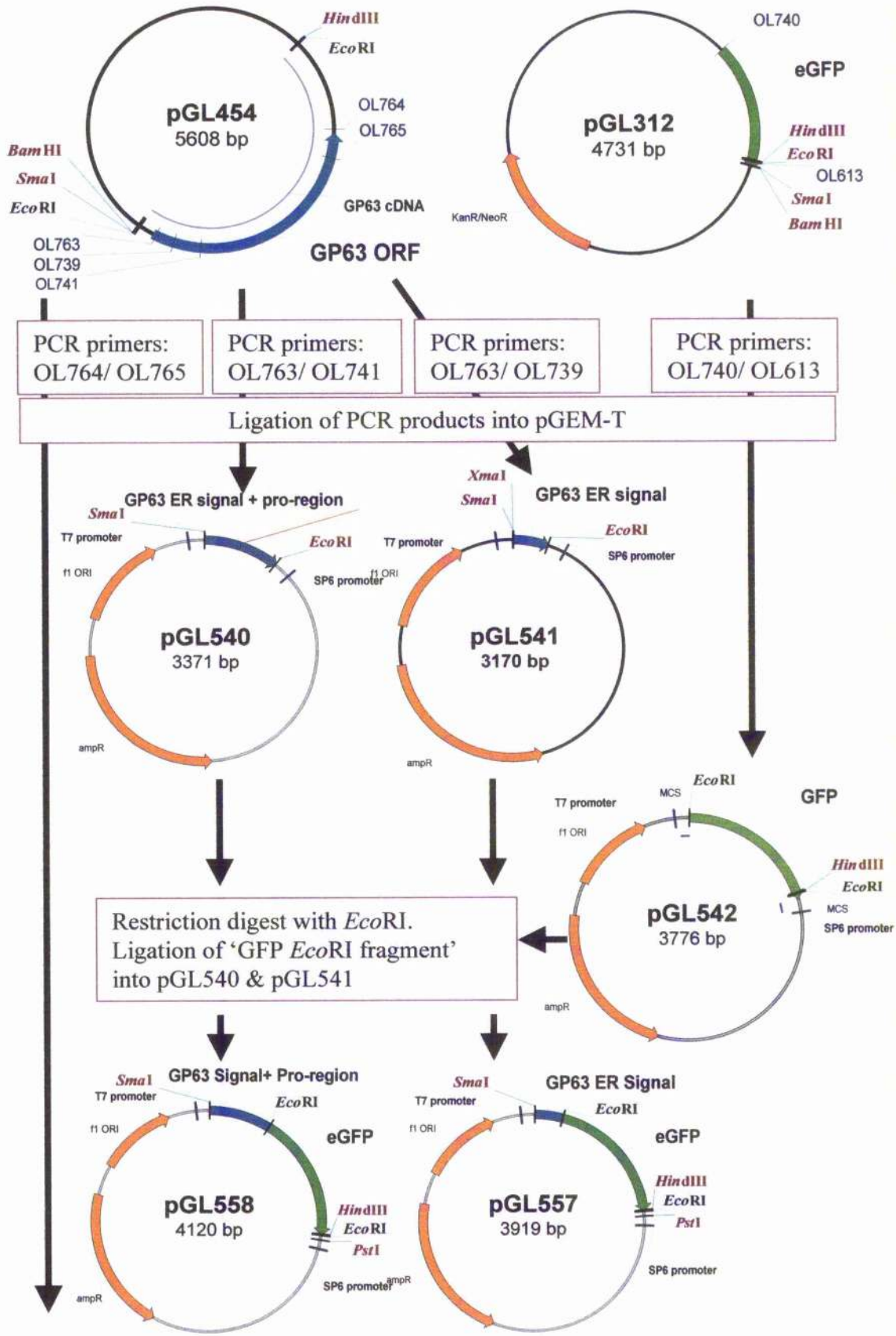
Figure 5.4: Schematic diagram of the GFP-GPI anchored fusion proteins ProGFP^{GPI} and GFP^{GPI}

The GFP fusion proteins incorporate domains from the GPI anchored protein GP63 fused to the N and C terminals of GFP. The models for the proteins ProGFP^{GPI} and GFP^{GPI} are shown, alongside GP63. The key includes a table indicating the number of amino acid residues incorporated from each region.

Figure 5.5: Schematic of the method used to clone episomally expressed GFP-GPI fusion proteins.

The plasmids pGL587 and pGL586 contain the ORFs coding for the GPI-anchored fusion proteins GFP^{GPI} and $ProGFP^{GPI}$, and were produced by multiple cloning steps as described in detail in the text.

Relevant restriction enzyme sites are indicated, as are the positions of relevant oligonucleotides.


pGL454
5608 bp
HindIII
EcoRI
OL764
OL765
GP63 cDNA
BamHI
SmaI
EcoRI
OL763
OL739
OL741
GP63 ORF
pGL312
4731 bp
OL740
eGFP
HindIII
EcoRI
OL613
SmaI
BamHI
KanR/NeoR
PCR primers: OL764/ OL765
PCR primers: OL763/ OL741
PCR primers: OL763/ OL739
PCR primers: OL740/ OL613
Ligation of PCR products into pGEM-T
GP63 ER signal + pro-region
SmaI
T7 promoter
EcoRI
f1 ORI
SP6 promoter
pGL540
3371 bp
ampR
XmaI
SmaI
GP63 ER signal
T7 promoter
EcoRI
SP6 promoter
f1 ORI
pGL541
3170 bp
ampR
EcoRI
MCS
T7 promoter
GFP
f1 ORI
HindIII
EcoRI
MCS
SP6 promoter
pGL542
3776 bp
ampR
Restriction digest with EcoRI.
Ligation of 'GFP EcoRI fragment'
into pGL540 & pGL541
SmaI
GP63 Signal+ Pro-region
T7 promoter
EcoRI
f1 ORI
eGFP
pGL558
4120 bp
HindIII
EcoRI
PstI
SP6 promoter
ampR
SmaI
GP63 ER Signal
T7 promoter
EcoRI
f1 ORI
eGFP
pGL557
3919 bp
HindIII
EcoRI
PstI
SP6 promoter
ampR

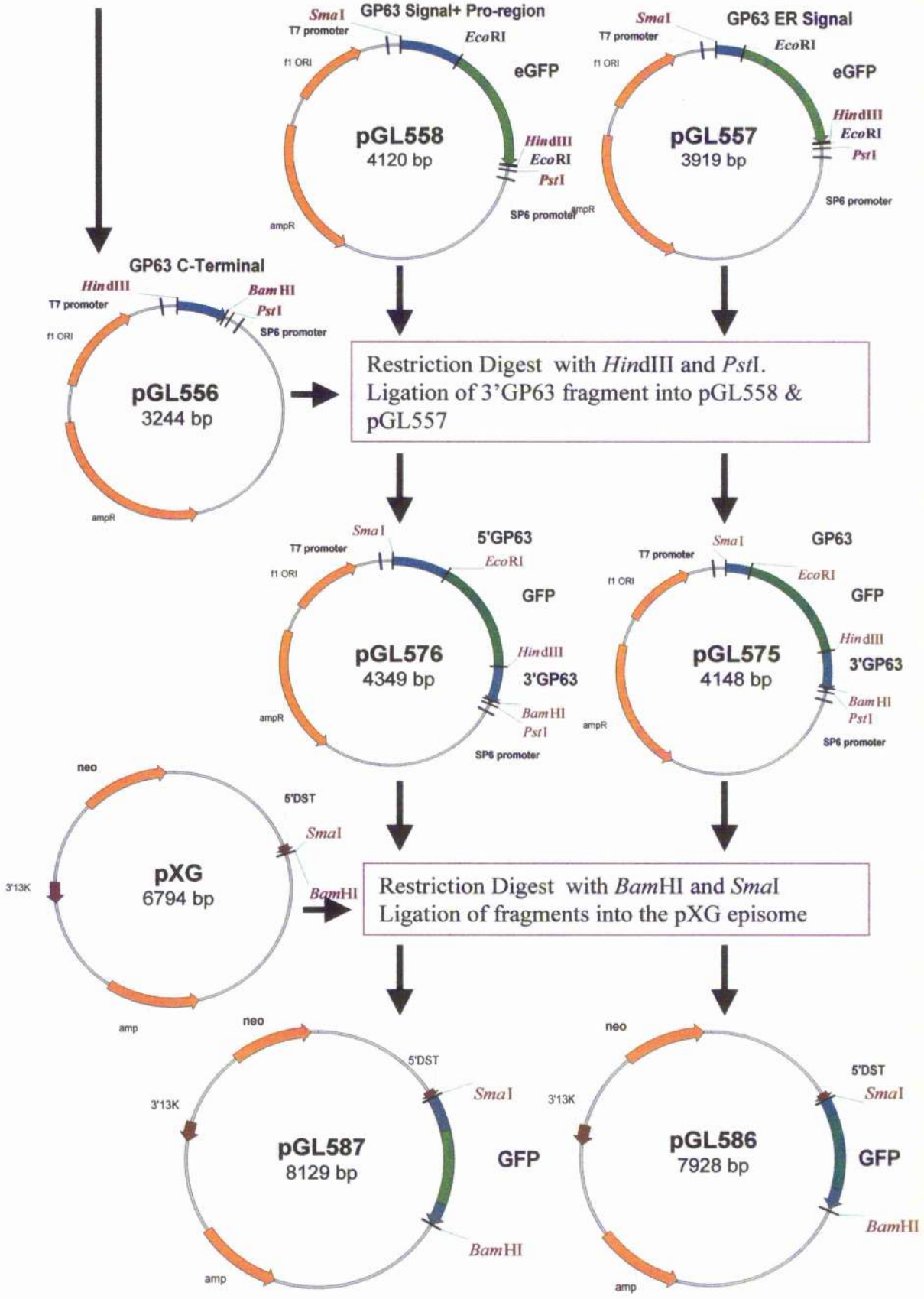
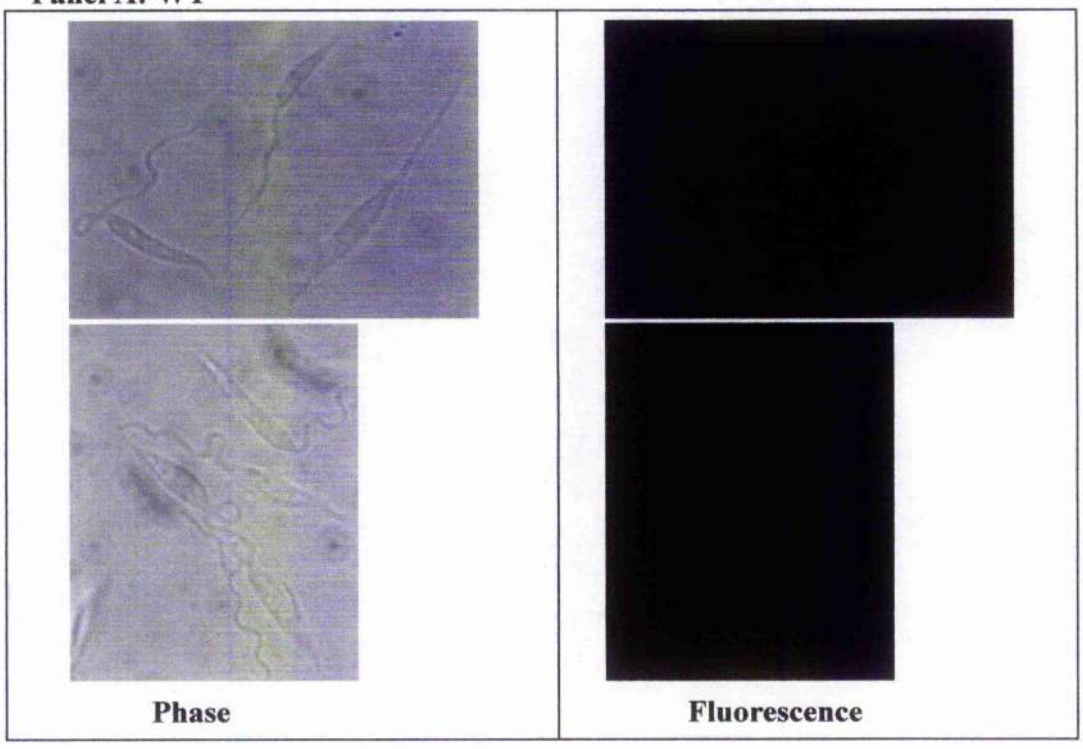

GP63 Signal+ Pro-region
SmaI
T7 promoter
EcoRI
f1 ORI
eGFP
pGL558
4120 bp
HindIII
EcoRI
PstI
ampR
SP6 promoter
GP63 ER Signal
SmaI
T7 promoter
EcoRI
f1 ORI
eGFP
HindIII
EcoRI
PstI
pGL557
3919 bp
SP6 promoter
ampR
GP63 C-Terminal
HindIII
T7 promoter
BamHI
PstI
f1 ORI
SP6 promoter
pGL556
3244 bp
ampR
Restriction Digest with HindIII and PstI.
Ligation of 3'GP63 fragment into pGL558 & pGL557
SmaI
T7 promoter
5'GP63
EcoRI
f1 ORI
GFP
pGL576
4349 bp
HindIII
3'GP63
ampR
BamHI
PstI
SP6 promoter
SmaI
T7 promoter
GP63
EcoRI
f1 ORI
GFP
HindIII
pGL575
4148 bp
3'GP63
BamHI
PstI
ampR
SP6 promoter
neo
5'DST
SmaI
3'13K
pXG
6794 bp
BamHI
amp
Restriction Digest with BamHI and SmaI
Ligation of fragments into the pXG episome
neo
5'DST
SmaI
3'13K
pGL587
8129 bp
GFP
BamHI
amp
neo
5'DST
SmaI
3'13K
pGL586
7928 bp
GFP
BamHI
amp

Figure 5.6: Fluorescence microscopy of GFP expressing cell lines.

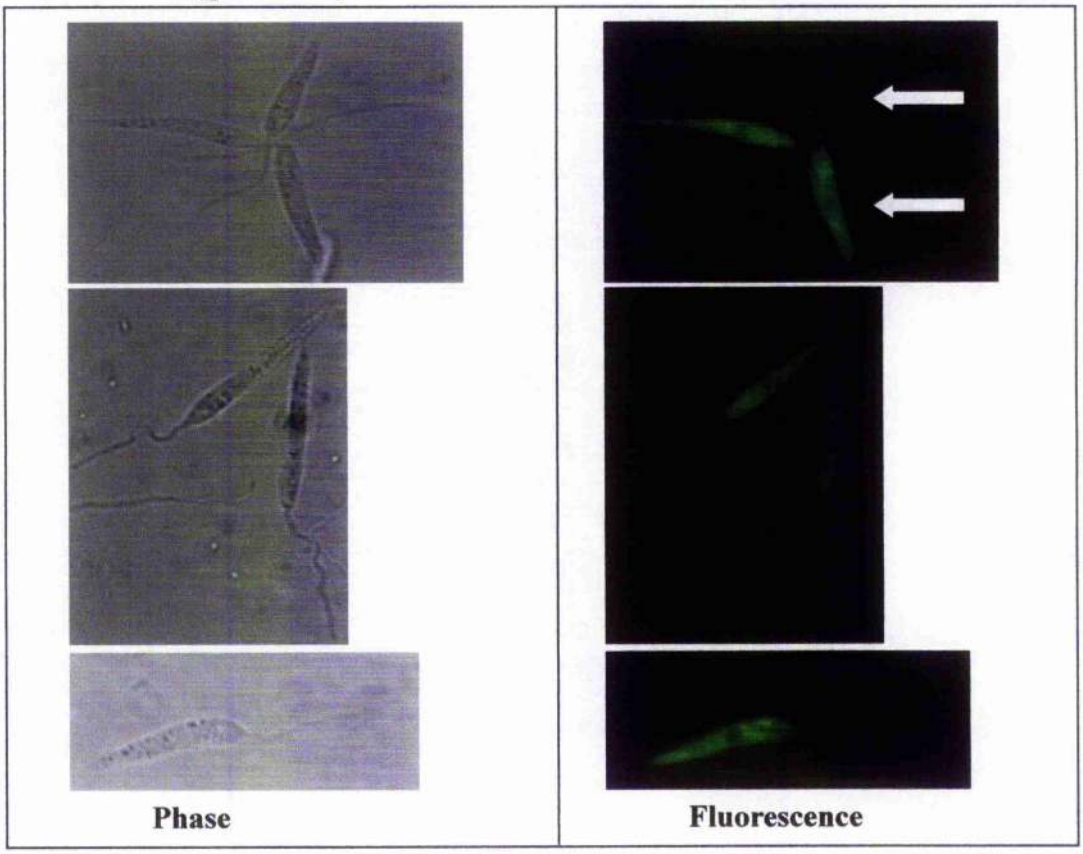
Live cells were treated with 0.005% NaN_3 to inhibit movement and examined by fluorescence microscopy using a Zeiss microscope. Filter settings were as those used for FITC labelled cells. Images were captured using a Hamamatsu digital camera, and visualised using Openlab (Improvision).

Arrows indicate cells for comparison which have variable levels of fluorescence.

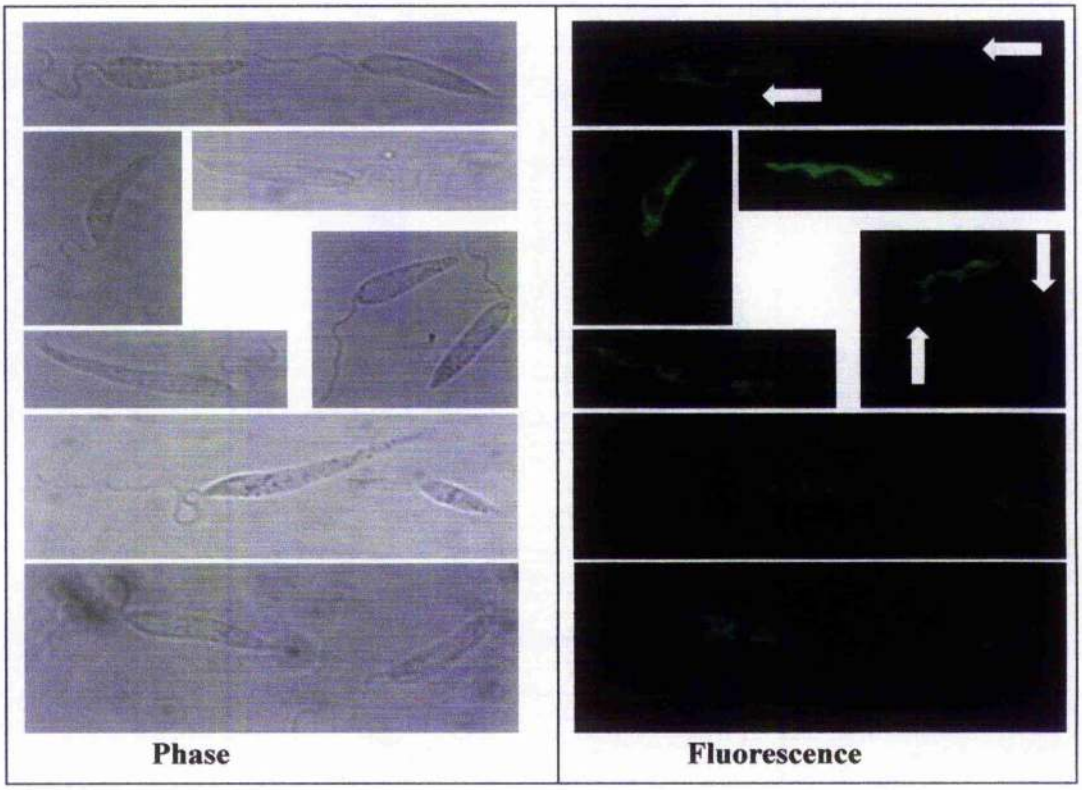
Panel A: WT



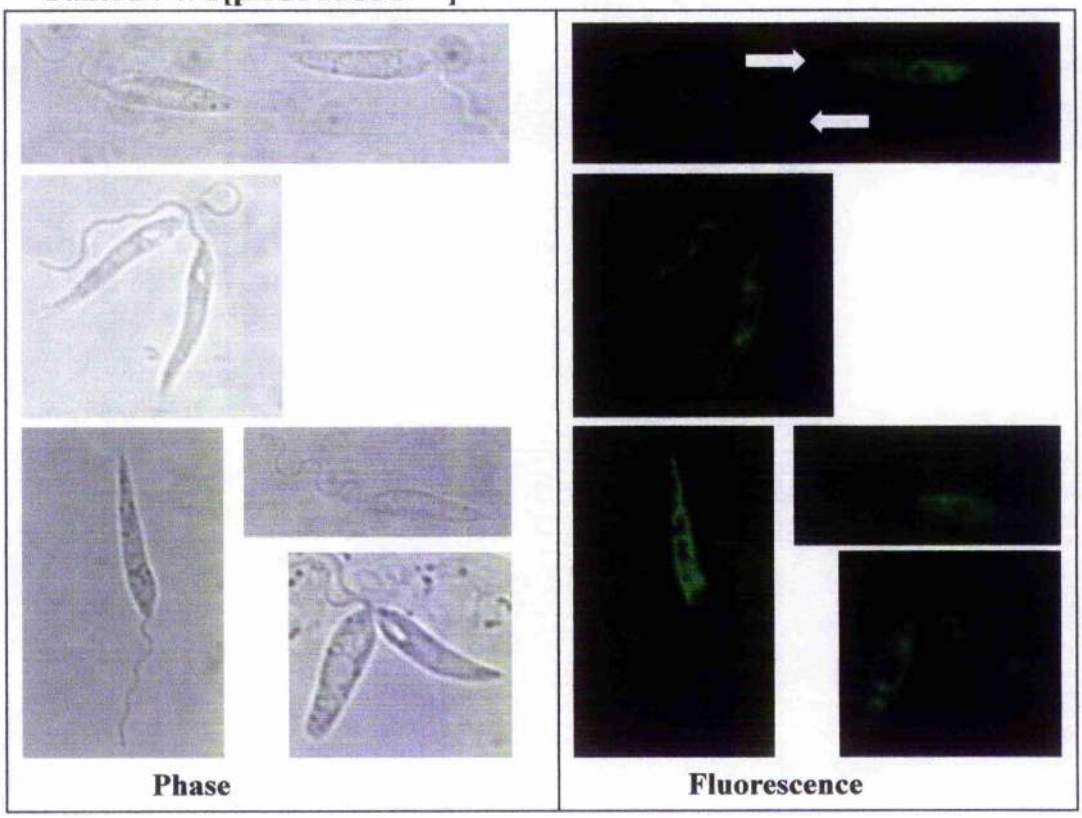
Panel B: WT[pXGGFP]



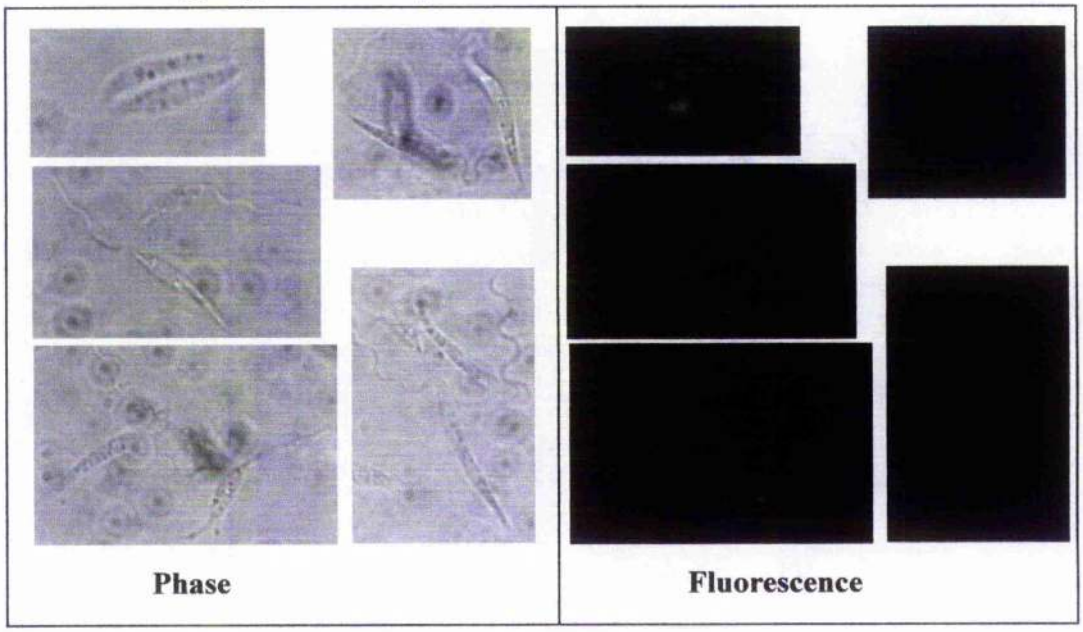
Panel C: WT[pXGGFP^{GPI}]



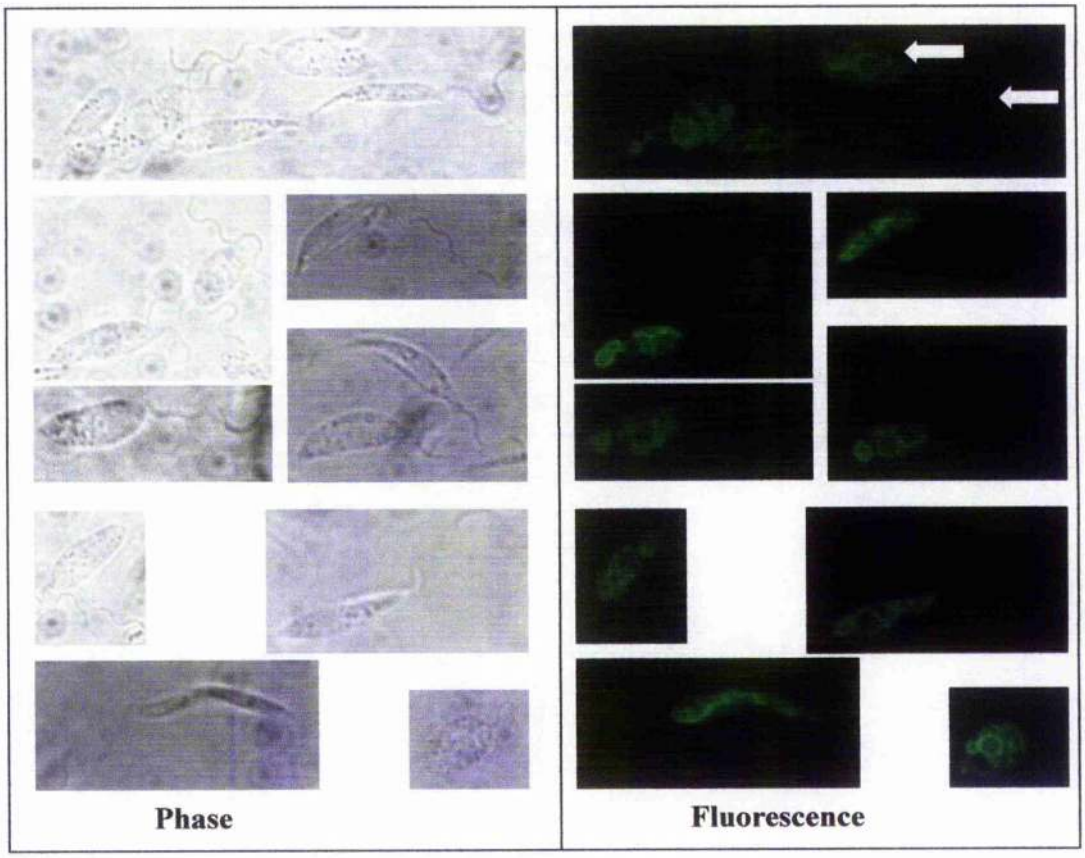
Panel D: WT[pXGProGFP^{GPI}]



Panel E: Δ*gpi8*[pXGGFP^{GPI}]



Panel F: Δ*gpi8*[pXGProGFP^{GPI}]



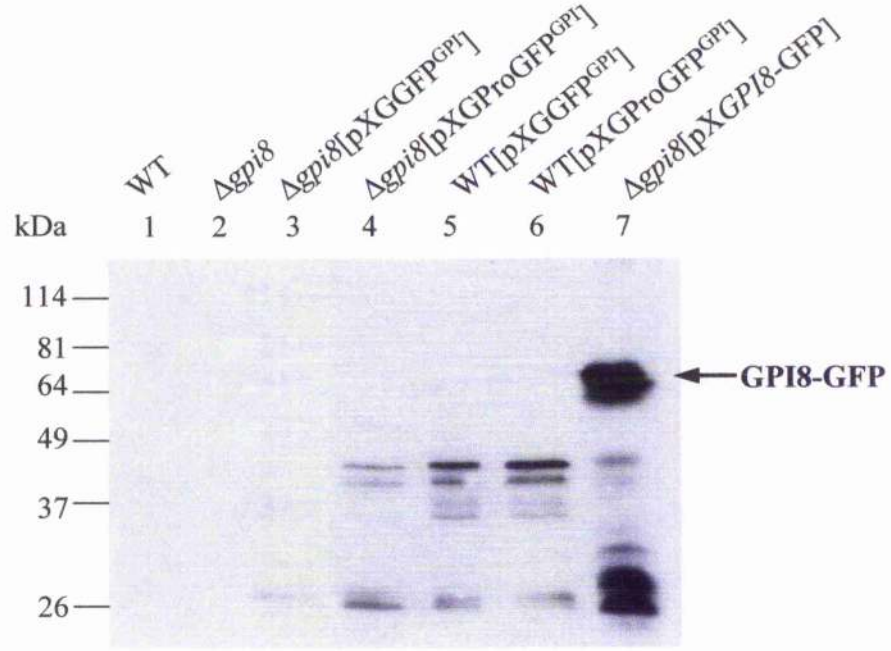


Figure 5.7: Analysis of GFP expression in cell lines expressing GPI-anchored GFP.

Cell lysates were prepared from the cell lines WT, Δ*gpi8*, Δ*gpi8*[pXGGFP^{GPI}], Δ*gpi8*[pXGProGFP^{GPI}], WT[pXGGFP^{GPI}], WT[pXGProGFP^{GPI}], and Δ*gpi8*[pX*GPI8*-GFP]. These were subjected to SDS-PAGE and electrophoresed at $1x10^7$ cell equivalents per lane, and electroblotted. The blot was exposed to a JL-8 GFP monoclonal antibody (Clontech), at a dilution of 1:1000. The 72 kDa GPI8-GFP fusion protein is indicated.

Figure 5.8: Analysis of GPI-anchored proteins by PI-PLC treatment and TritonX-114 extraction of GFP expressing cell lines.

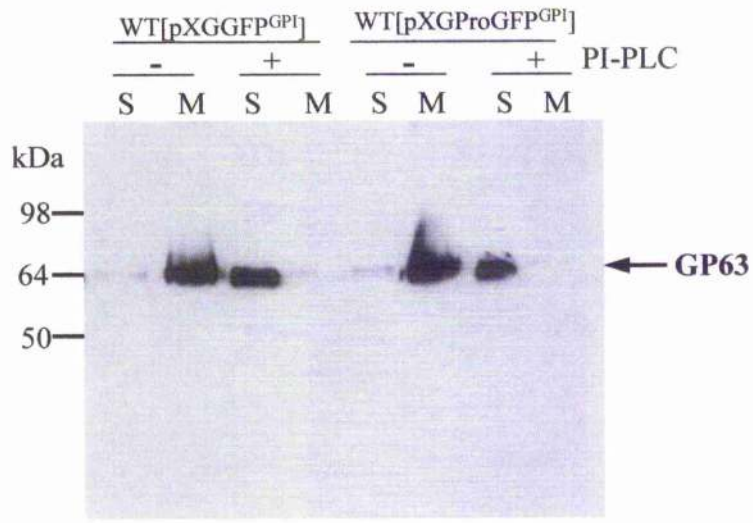
10^8 cells were pelleted from the cell lines WT[pXGGFP^{GPI}], WTpXGProGFP^{GPI}], Δ*gpi8*[pXGGFP^{GPI}], and Δ*gpi8*[pXGProGFP^{GPI}].

Cells were lysed and treated with or without PI-PLC prior to Triton X-114 fractionation into soluble (S) and membrane-associated (M) fractions. Samples were subjected to 12% SDS-PAGE and electroblotted.

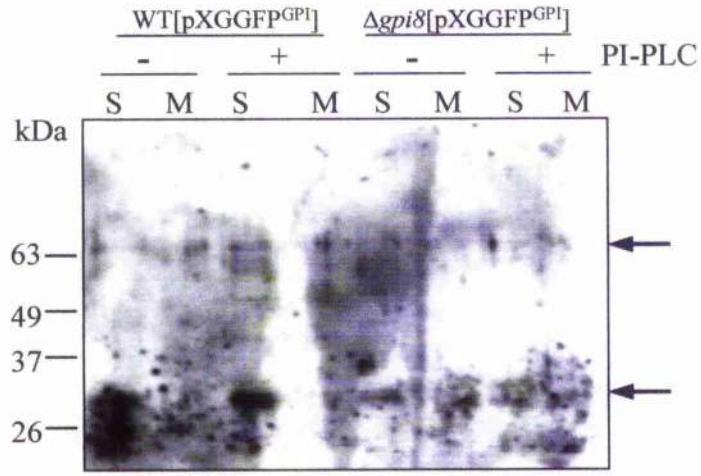
Panel A) The blot was exposed to a *L. major* GP63 monoclonal antibody at a dilution of 1:50. The 63kDa GP63 protein is indicated.

Panel B and C) The blots were exposed to the Clontech JL-8 GFP monoclonal antibody at a dilution of 1:1000. The arrows indicate possible GFP proteins and associated degradation products.

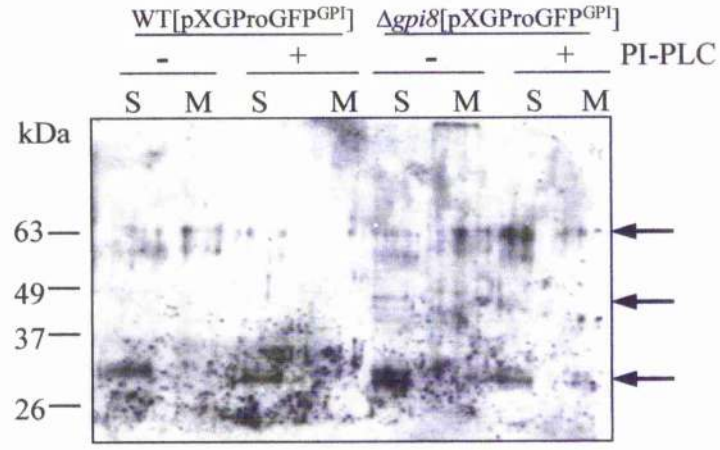
A


WT[pXGGFP^GPI]
WT[pXGProGFP^GPI]
-
+
-
+
PI-PLC
S M S M S M S M
kDa
98
64
50
GP63

B


WT[pXGGFP^GPI]
Δgpi8[pXGGFP^GPI]
-
+
-
+
PI-PLC
S M S M S M S M
kDa
63
49
37
26

C


WT[pXGProGFP^GPI]
Δgpi8[pXGProGFP^GPI]
-
+
-
+
PI-PLC
S M S M S M S M
kDa
63
49
37
26

Chapter 6

Discussion

The aims of this work were the continuation of the characterisation of GPI8, the identification of novel proteins associated with GPI biosynthesis or trafficking, and the analysis of the trafficking of GPI, and non-GPI-anchored protein in *L. mexicana*.

The catalytic active sites of *L. mexicana* GPI8 were identified as C216 and H174. This result is fully consistent with the situation in the yeast and mammalian homologues, where the active sites were identified as C199 and H157, and Cys206 and H164 respectively (Meyer *et al.*, 2000; Ohishi *et al.*, 2000). This demonstrates the high level of conservation at the position of the catalytic dyad within the GPI8 subfamily of the C13 cysteine proteases. The GPI8 of *L. mexicana* was also demonstrated to be part of a larger complex, again consistent with the situation in higher eukaryotes. This suggests that a high level of similarity exists between the GPIT complexes in higher eukaryotes and trypanosomatids. However important differences also exist, such as the soluble nature of the *L. mexicana* and *T. brucei* GPI8 (Hilley *et al.*, 2000; Sharma *et al.*, 2000), compared to the yeast and mammalian homologues which have a transmembrane domain.

The GPI8 amino acid residue C94 was identified as functionally important, and the protein GPI8^{C94G} demonstrated to be dysfunctional with respect to GP63 GPI-anchor addition. The homologous mutation in the human GPI8 also results in a decrease in transamidase activity (Ohishi *et al.*, 2000). It is possible that this residue has some catalytic activity, other than involvement with the transamidation mechanism. Alternatively the residue may be required for maintenance of tertiary structure of the GPI8 protein or the GPIT complex. The latter is possible as the residue is conserved amongst the GPI8 subfamily (but not the C13 subfamily) and may form a disulphide bond with another member of the GPIT complex. Similarly epitope tagging of GPI8 at a position C-terminal of the identified active site residues also resulted in an inactive GPIT complex. The reason for this was not clear but it is possible that insertion of the 9 amino acid peptide resulted in conformational changes to the GPI8 protein. Co-

precipitation experiments with cell lines expressing GPI8-TY^{NdeI} or a tagged form of GPI8^{C94G}, and comparison with cell lines expressing either the inactive or the fully functional GPI8 would be interesting.

Photo cross-linking experiments have previously demonstrated that GPI8 interacts directly with the pro-protein (Spurway *et al.*, 2001; Vidugiriene *et al.*, 2001), but it is not known how other members of the GPIT complex interact. As both *L. mexicana* and *T. brucei* GPI8 lack a transmembrane domain common in higher eukaryotes it is likely that interaction with other GPIT complex members allows the GPI8 protein to associate with the ER membrane. It would therefore be interesting to examine in-depth the protein-protein interactions of the different GPIT complex members. It is also possible that the GPIT complex of trypanosomatids has a different complexity from that of higher eukaryotes with a different number of members, or the proteins involved having differing roles.

The identification of other GPIT complex members seems an obvious and interesting area for further research. The presence in the *L. major* genome database of a putative GAA1 homologue (Eisenhaber *et al.*, 2001), provides a suitable starting point for further analysis. The present difficulties associated with the detection of GPI8 in WT cells is thought to be due to the low level of expression of the protein. However, isolation of a second complex member and production of antibodies against this may provide different results. The detection of the complex intracellularly by immune-fluorescence or direct immune-precipitation of the complex would allow the comparison of the GPIT complex of higher and lower eukaryotes.

The novel method devised for screening for GPI biosynthesis and trafficking mutants was unsuccessful. Technical difficulties, associated with variations in GFP fluorescence in different cells within a single population, suggested that the method would not be suitable for a protracted screening process. The failure to produce a GFP GPI-anchored on the cell surface suggests that the signals required for anchor addition and trafficking are complex. Other recent studies in trypanosomatids reported similar difficulties in producing GPI-anchored GFP (Bohme and Cross, 2002). The report that a successful GPI-anchored GFP chimera was produced utilising the signal attachment sequences from GP63 (Ghedin *et al.*, 2001), as attempted in this study, only serves to underline

the complexities involved in GPI-anchor addition. The failure to produce surface anchored GFP in this study may be explained by the fact that the trafficking of GPI-anchored proteins is complex, and may be mediated by a variety of unidentified trafficking and sorting signals (Nichols *et al.*, 2001). Similarly the precise requirements for the signals for GPI anchor attachment appear to be different for individual proteins (Aceto *et al.*, 1999), and it is possible that this is associated with the signals required to mediate the interaction with the GPIT complex.

It is interesting that the fate of the GFP, in both WT and the Δ*gpi8* cells, was different from that of unanchored GP63 in the Δ*gpi8* cell line. The unanchored GFP was retained within the ER, whilst the majority of unanchored GP63 was secreted. It is possible that the GFP was retained, due to a failure to remove the ER signal. This would have interesting implications for the workings of translocation machinery. Proteins are predicted to have their ER signal removed by the translocon machinery as they pass through the translocon pore, and thus would not be expected to be affected by a non-native protein sequence C-terminal to the cleavage point. Alternatively the failure to secrete GFP from the cells, could suggest that unanchored GP63 contains signals which specifically direct its transport from the cell.

In mammalian cells, the use of GFP chimeras and confocal microscopy coupled with photobleaching techniques have provided exciting methods to directly analyse the processes and dynamics of vesicular transport within live cells (Lippincott-Schwartz *et al.*, 2000). In *L. mexicana* the multi-vesicular tubule, a novel compartment has been identified using a GFP-DPMS chimera (Ilgoutz *et al.*, 1999a; Mullin *et al.*, 2001). The sorting of VSG has been examined in *T. brucei* by fluorescence microscopy, biotinylation and electron microscopy, and has been concluded to occur in multiple membrane compartments (Grunfelder *et al.*, 2002). The production of a GFP chimera associated with the complete GP63 protein would allow the direct study and comparison of the trafficking pathways in both WT and Δ*gpi8* cells, using similar methods, and would provide an interesting comparison of the trafficking of a GPI-anchored versus non-anchored protein.

Some differences exist in the secretory trafficking pathway between mammalian and trypanosomatids. EM microscopy suggests that the ERGIC identified in mammalian cells and the ER to Golgi transport in *L. mexicana* differ, as does the ultrastructure of the TGN compared with the trans-Golgi of *L. mexicana* (Weise *et al.*, 2000). This study identified that the inhibitor Brefeldin A, associated with disruption of the Golgi apparatus in yeast and mammalian cells, had no discernible affect in *L. mexicana*. These differences suggest that the continued study of trafficking in these organisms may provide potential new drug targets.

Detailed comparison of the trafficking of GP63 in the WT and Δ*gpi8* cell lines demonstrated that in WT cells GP63 is trafficked to the cell surface, and during this trafficking the nascent protein is processed through two identifiable intermediate forms. In the Δ*gpi8* cell line the nascent protein is N-glycosylated and secreted directly from the cell with no further processing. The abolition of GPI anchor addition therefore effects both the trafficking and processing of GP63 in *L. mexicana*. Loss of N-glycosylation inhibited the secretion of GP63 in the Δ*gpi8* cell line. In other systems loss of GPI anchoring resulted in the retention of the protein within the ER, and it is thought that the GPI anchor itself may be an important signal in the onward trafficking of proteins (Field *et al.*, 1994; Doering and Schekman, 1997). It has also been reported that in the absence of other targeting signals N-glycans can act as signals for protein trafficking (Gut *et al.*, 1998). In this study neither the loss of GPI-anchor addition nor the loss of N-glycosylation resulted in the intracellular retention of GP63. However, loss of both GPI-anchor addition and N-glycosylation inhibited secretion of the nascent form of GP63 into the medium. This indicates that in the absence of a GPI anchor, N-glycans are important in directing the trafficking of GP63 from the cell.

In this system, it seems that a small proportion of the nascent non-anchored GP63 is secreted from the WT cells, and suggests that GP63 may be trafficked by two separate pathways in WT cells. The Δ*gpi8* cell line is able to utilise the second pathway in the absence of GPI anchoring. It is proposed that in WT cells two pathways exist for the trafficking of GP63 from the cell. Trafficking is either by a classical pathway whereby GP63 is N-glycosylated, GPI-anchored and then undergoes further modification during transport to the cell surface, or a direct secretion pathway whereby non-GPI-anchored

GP63 is secreted rapidly from the cell without subsequent modification. It appears that the requirement for entry into the first pathway is the presence of a GPI anchor, whilst the alternate pathway appears to require both the absence of a GPI anchor and presence of N-glycans. GPI-anchoring in *Leishmania* does affect the forward trafficking of GPI-anchored proteins. However, this study does not address whether GPI anchors act directly as a signal for the forward transport of proteins, or if they play an active role in protein transport.

GPI-anchored proteins become insoluble to detergent extraction during trafficking through the secretory pathway forming detergent resistant membranes (DRMs). Lipid-microdomains occur in protozoa (Nolan *et al.*, 2000; Denny *et al.*, 2001; Ralton *et al.*, 2002). DRMs can be isolated from *L. major*, and are enriched in characteristic eukaryotic lipid raft components; inositol phosphorylceramide, sterols such as ergosterol and GPI-anchored molecules (both GP63 and LPG). In *L. major* GP63 is rapidly incorporated into DRMs suggesting that rafts may form in the ER of *Leishmania* as in yeast (Denny *et al.*, 2001). Studies in *L. mexicana* suggested that GP63 was incorporated into DRMs with slow kinetics, suggesting incorporation in a late secretory compartment and indicating a discrepancy with the situation in *L. major* (Ralton *et al.*, 2002).

Lipid rafts appear to be extremely relevant to the forward trafficking of GPI proteins in *Leishmania*. Continued investigation into this process would be extremely interesting, such as the use of ergosterol inhibitors to inhibit the formation of lipid rafts. Study of the Δ*gpi8* cell line would continue to provide useful insights into the trafficking of GPI proteins in *Leishmania*.

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