The EMBO Journal Vol.18 No.13 pp.3643–3654, 1999

Glycosylphosphatidylinositol biosynthetic enzymes are localized to a stable tubular subcompartment of the endoplasmic reticulum in *Leishmania mexicana*

Steven C.Ilgoutz[1], Kylie A.Mullin[1], Bridget R.Southwell[2] and Malcolm J.McConville[1,3]

[1]Departments of Biochemistry and Molecular Biology, and [2]Anatomy and Cell Biology, University of Melbourne, Royal Parade, Parkville, Victoria 3052, Australia

[3]Corresponding author
e-mail: m.mcconville@biochemistry.unimelb.edu.au

Glycosylphosphatidylinositols (GPI) are essential components in the plasma membrane of the protozoan parasite *Leishmania mexicana*, both as membrane anchors for the major surface macromolecules and as the sole class of free glycolipids. We provide evidence that *L.mexicana* dolichol-phosphate-mannose synthase (DPMS), a key enzyme in GPI biosynthesis, is localized to a distinct tubular subdomain of the endoplasmic reticulum (ER), based on the localization of a green fluorescent protein (GFP)–DPMS chimera and subcellular fractionation experiments. This tubular membrane (termed the DPMS tubule) is also enriched in other enzymes involved in GPI biosynthesis, can be specifically stained with the fluorescent lipid, BODIPY-C_5-ceramide, and appears to be connected to specific subpellicular microtubules that underlie the plasma membrane. Perturbation of microtubules and DPMS tubule structure *in vivo* results in the selective accumulation of GPI anchor precursors, but not free GPIs. The DPMS tubule is closely associated morphologically with the single Golgi apparatus in non-dividing and dividing cells, appears to exclude luminal ER resident proteins and is labeled, together with the Golgi apparatus, with another GFP chimera containing the heterologous human Golgi marker β1,2-*N*-acetylglucosaminyltransferase-I. The possibility that the DPMS-tubule is a stable transitional ER is discussed.

Keywords: dolichol-phosphate-mannose synthase/endoplasmic reticulum/Golgi/*Leishmania*

Introduction

Many proteins entering the secretory pathway of eukaryotic cells are modified with a glycosylphosphatidylinositol (GPI) anchor, which is required for membrane attachment and may play a role in regulating the intracellular transport and biological properties of the protein (reviewed in McConville and Ferguson, 1993; Stevens, 1995; Takeda and Kinoshita, 1995). Non-protein-linked or free GPIs may also be abundant membrane constituents, although the function of these glycolipids in animal cells is unclear (van't Hof *et al*., 1995). GPI anchor precursors are assembled in the endoplasmic reticulum by the sequential transfer of monosaccharides and ethanolamine-phosphate (EtN-P) to phosphatidylinositol (PI), to form the minimal structure EtN-P-Man_3GlcN-PI. The mature glycolipid precursor is then transferred to nascent proteins containing an appropriate C-terminal GPI signal sequence. The initial steps in GPI anchor precursor biosynthesis (up to the formation of GlcN-PI) take place on the cytoplasmic side of the ER, while the latter steps, including the addition of the third mannose residue and EtN-P occur in the ER lumen (Vidugiriene and Menon, 1993, 1994; Takahashi *et al*., 1996; Watanabe *et al*., 1996). Interestingly, the first enzyme in this pathway is present in a multiprotein complex which appears to be localized to the rough ER in animal cells (Watanabe *et al*., 1996). Whether other steps in GPI biosynthesis are segregated into subdomains of the ER is not known.

GPI biosynthesis is highly upregulated in many parasitic protozoa that cause important diseases in humans, which is reflected in the abundance of GPI-anchored glycoproteins on their cell surfaces (McConville and Ferguson, 1993; Ferguson, 1997). These organisms have thus proved to be useful experimental systems for delineating the steps involved in GPI biosynthesis. Because GPI anchors are required for the attachment of major cell surface virulence factors to the plasma membrane, information on GPI biosynthesis in these organisms may also lead to the identification of new drug targets. Our laboratory has focussed on the sandfly-transmitted parasites belonging to the genus *Leishmania*, which alternate between a flagellated promastigote stage in the digestive tract of the sandfly vector and a non-motile amastigote stage that invades and proliferates within macrophages of the mammalian host. The cell surface of the promastigote stage is coated by a number of GPI-anchored glycoproteins, a GPI-anchored lipophosphoglycan (LPG) and a family of free GPIs, termed glycoinositol-phospholipids (GIPLs) (McConville and Ferguson, 1993). LPG is the major promastigote surface macromolecule and is required for parasite survival in both the sandfly and mammalian hosts (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). The GIPLs are also abundant cell surface components and probably form a tightly packed glycocalyx immediately above the plasma membrane (McConville and Blackwell, 1991). Although the function of these glycolipids is not known, they appear to be essential for parasite growth (Ilgoutz *et al*., 1999).

Recent studies in *Leishmania mexicana* and *Leishmania major* suggest that the LPG anchor and GIPL precursors are assembled in the same way as the protein anchor precursors, by the sequential transfer of monosaccharides to PI (Proudfoot *et al*., 1995; Smith *et al*., 1997; Ralton and McConville, 1998). The protein and LPG anchor precursors are assembled on the same pool of PI molecular species, although these pathways diverge beyond the common precursor, Man_1GlcN-PI (Ralton

and McConville, 1998). Specifically, the first committed intermediate in LPG anchor biosynthesis is synthesized by the addition of an α1-3 (rather than a α1-6-linked) mannose residue to this precursor. It is not known whether the α1-3-linked mannose residue is added in the ER, where other GPI-specific mannosyltransferases are thought to be located (Vidugiriene and Menon, 1993), or in the Golgi apparatus, where at least one of the galactosyltransferases involved in elongating the LPG anchor precursors is located (Huang and Turco, 1993; Ha *et al*., 1996). While the structures of the protein and LPG anchor are highly conserved in different *Leishmania* spp., the structures of the GIPLs are more polymorphic (McConville and Ferguson, 1993; Zawadzki *et al*., 1998). In *L.mexicana*, the GIPLs have the same Man_3GlcN-PI backbone as the protein anchors, but are modified by the LPG anchor-specific α1-3-linked mannose residue (hybrid-type GIPLs; McConville *et al*., 1993). Intriguingly, we have shown that these abundant glycolipids are assembled on a distinct pool of PI precursors from those incorporated into either the LPG or protein anchor precursors, indicating that they are products of a distinct biosynthetic pathway rather than excess intermediates of protein and LPG anchor biosynthesis (Ralton and McConville, 1998). These studies suggest that *L.mexicana* promastigotes contain at least two distinct pools of GPI precursors, which are differentially modified by a set of common mannosyltransferases. In order to account for the incorporation of distinct PI molecular species into these glycolipids, we have previously speculated that enzymes in one or more of these pathways may be segregated into distinct subdomains of the ER (Ralton and McConville, 1998).

To investigate the possible compartmentalization of GPI biosynthetic enzymes in the ER of *Leishmania*, we have expressed a functional fusion of GFP to the N-terminus of dolichol-phosphate-mannose synthase (DPMS). DPMS catalyzes the transfer of mannose from GDP–Man to dolichol-phosphate to form dolichol-phosphate-mannose (DPM), the immediate sugar donor for the GPI-specific mannosyltransferases that make the conserved backbone of the protein anchors (Menon *et al*., 1990; Orlean, 1990), but not for the putative LPG anchor and GIPL-specific α1-3 mannosyltransferase (Ilgoutz *et al*., 1999). As GPI biosynthesis is the major biosynthetic pathway to utilize DPM in *L.mexicana* (Ilgoutz *et al*., 1999), this enzyme should provide a marker for the location of DPM-dependent GPI biosynthetic reactions. The GFP–DPMS chimera was localized to a tubular compartment that may correspond to a novel subdomain of the ER. This compartment was also identified in non-transformed cells and appears to contain a range of both DPM-dependent and -independent GPI biosynthetic activities. These data provide important new insights into the organization of the secretory pathway of these parasites and a further example of how metabolic processes may be segregated into subdomains of the ER in eukaryotic cells.

Results

DPMS is a marker for a distinct tubular membranous subcompartment

We have recently cloned the *L.mexicana* gene for DPMS which is predicted to encode a 29 kDa membrane protein

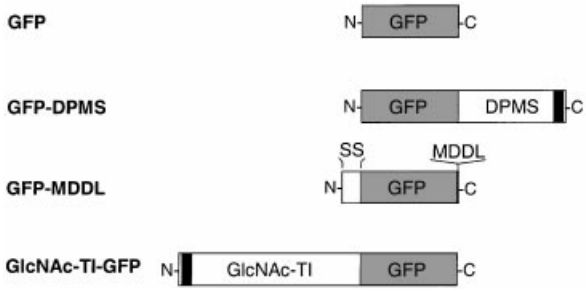

Fig. 1. Structure of GFP–chimeras used in this study. GFP fusions with the N-terminus of *L.mexicana* DPMS, the C-terminus of human *N*-acetylglucosaminyltransferase (GlcNAc-T1), and the *T.brucei* BiP N-terminal signal sequence (SS) and C-terminal ER retention signal (MDDL) are shown. The transmembrane domains of DPMS and GlcNAc-T1 are represented by a black bar.

that is anchored to the cytosolic side of intracellular membranes via a C-terminal hydrophobic domain (Ilgoutz *et al*., 1999). The subcellular localization of this enzyme was investigated by expressing a fusion of GFP with the N-terminus of DPMS (GFP–DPMS) in *L.mexicana* promastigotes (Figure 1). In initial experiments we found that GFP–DPMS was catalytically active when expressed in both *Escherichia coli* and in *L.mexicana* promastigotes, as indicated by a 50% increase in enzyme activity over endogenous wild-type levels in the latter (unpublished data). A similar increase in activity was observed when untagged DPMS was expressed in promastigotes from the episomal vector pX, suggesting that levels of expression of this enzyme are tightly regulated. To assist with the localization of this and other GFP chimeras, the surface glycocalyx of live promastigotes containing mannose-rich glycoproteins and LPG were labeled with concanavalin A conjugated with tetramethylrhodamine (ConA–TRITC; Sigma) (Figure 2a–m). This probe stains the entire plasma membrane, including the anteriorly located flagellum and flagellar pocket, a specialized invagination of the plasma membrane which is the sole site of exocytosis and endocytosis in these parasites (Overath *et al*., 1997). Unexpectedly, confocal scanning laser microscopy revealed that the GFP–DPMS chimera was confined to a distinct tubular membrane in *L.mexicana* promastigotes. This tubule (termed the DPMS tubule) always abutted the flagellar pocket and extended along the anterior–posterior axis of the promastigotes (Figure 2a and b). Optical sections spanning the depth of the cell confirmed that this compartment had a tubular rather than a cisternal structure (Figure 2e–i). Loops were frequently observed at the ends or along the length of the tubule structure (e.g. Figure 2b) and were sometimes seen to move along the tubule indicating a highly dynamic structure. The distribution of the GFP–DPMS chimera differed from that of native GFP, which was distributed throughout the cytoplasm (Ha *et al*., 1996; unpublished data), and a GFP construct containing an N-terminal signal sequence and the C-terminal tetrapeptide, MDDL, which acts as a ER retention signal in *Trypanosoma brucei* (Bangs *et al*., 1993) (Figure 2c). The latter construct was localized to the nuclear envelope and a transcellular reticulum that was often closely associated with the plasma membrane, consistent with a localization in the lumen of the bulk ER (Figure 2c, k and m). Deletion of the C-terminal tetrapeptide prevented detectable accu-

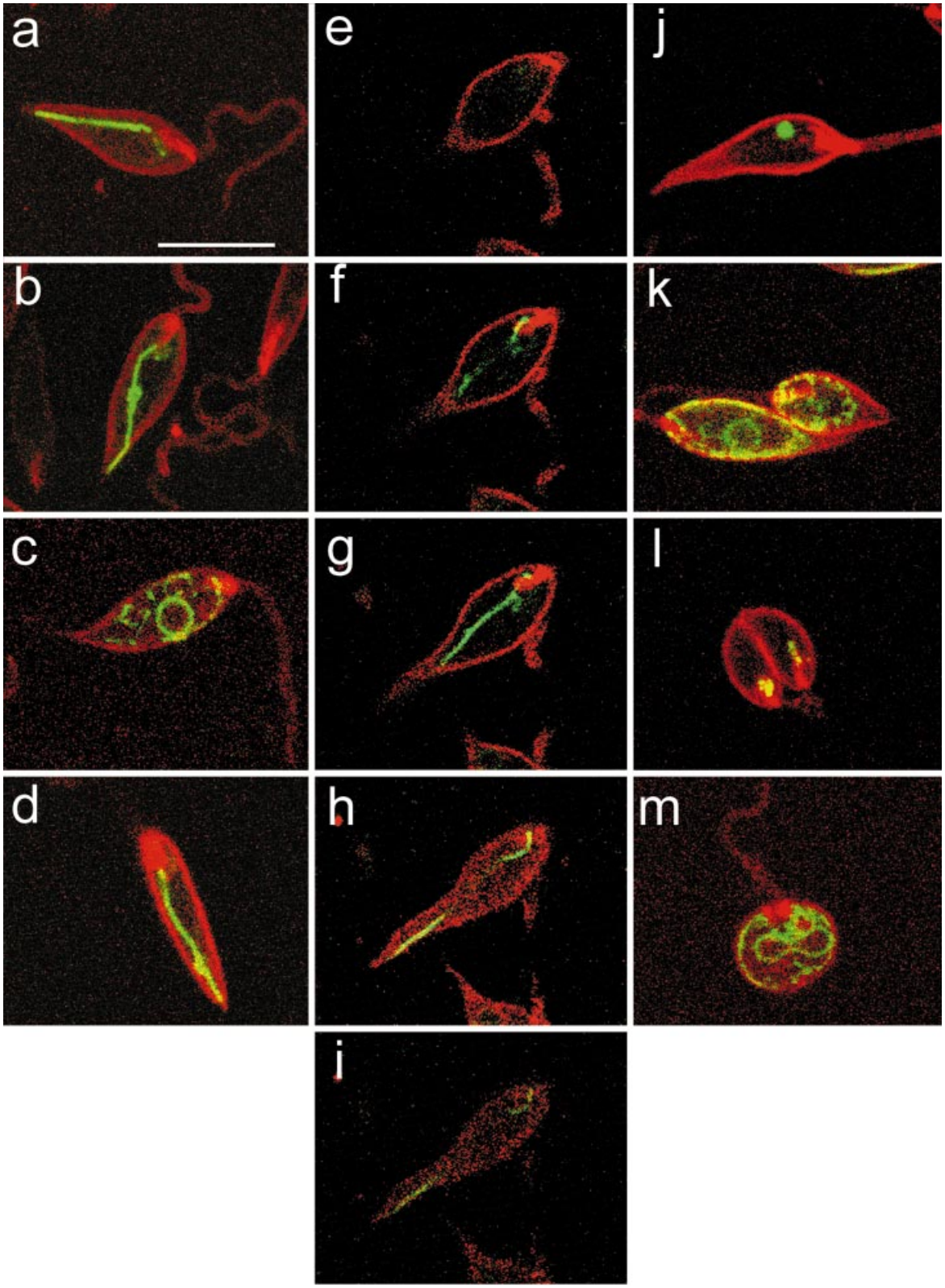

Fig. 2. Identification of a novel tubular organelle in *L.mexicana*. Promastigotes expressing various GFP chimeras or non-transformed cells which had been labeled with BODIPY-C_5-Cer (green fluorescence) were incubated briefly in the presence of ConA–TRITC (red fluorescence) prior to being analyzed by scanning confocal microscopy. [(**a**) and (**b**)] Representative images of promastigotes expressing GFP–DPMS. (**c**) Promastigotes expressing GFP–MDDL. (**d**) Promastigotes incubated with BODIPY-C_5-Cer for 10 min then backextracted with BSA for 2 h. (**e**–**i**) Five 0.5 μm optical sections taken through the same cell expressing the GFP–DPMS chimera. Promastigotes expressing the GFP–DPMS (**j**) and GFP–MDDL (i) after 5 min treatment with 15 μg/ml thioridazine. Dividing promastigotes expressing GFP–DPMS (**l**) and GFP–MDDL (**m**). Scale bar, 10 μm.

mulation of this GFP chimera in the ER (unpublished data), demonstrating that the trypanosome retention signal is functional in *Leishmania*. Finally, the distribution of GFP–DPMS fluorescence was markedly different from the previously reported distribution of a GFP–chimera containing an endogenous Golgi protein, LPG1p, which was localized to a single Golgi stack proximal to the flagellar pocket (Ha *et al*., 1996).

The DPMS tubule could also be detected in non-transformed cells which had been labeled with the fluorescent ceramide analogue, BODIPY-C_5-Cer. This analogue has a higher critical micellar concentration than that of natural ceramide (Pagano *et al*., 1989), and therefore rapidly inserts into the plasma membrane and intracellular membranes, including the ER, when incubated with promastigotes at 27°C. However, when cells were incubated further in medium containing defatted bovine serum albumin (BSA) to remove fluorescent lipid at the cell surface, the BODIPY-C_5-Cer accumulated in a membrane tubule that was indistinguishable from the DPMS tubule (Figure 2d). No fluorescence remained in either the ER or the region near the flagellum pocket containing the Golgi apparatus (Figure 2d). This accumulation was associated with the modification of the BODIPY-C_5-Cer with an inositol-phosphate headgroup to form the short chain analogue of inositolphosphoceramide (IPC), the major sphingolipid of these parasites (K.A.Mullin and M.J. McConville, unpublished data). The accumulation of BODIPY-Cer in the DPMS tubule could thus reflect its association with specific lipids in this compartment and/or inhibition of further transbilayer movements if the addition of the inositol-phosphate headgroup occurs in the lumen of the DPMS tubule. In either case, this observation suggests that the DPMS tubule exists in non-transformed cells and that it is likely to have a lipid composition distinct from other intracellular membranes.

The DPMS tubule is associated with the subpellicular microtubules

The organization of tubular membrane networks in many eukaryotes is maintained by specific associations with either the microtubule or actin cytoskeletons. Trypanosomatid parasites lack a transcellular cytoskeleton, but contain a cage-like array of subpellicular microtubules that are extensively cross-linked with each other and the overlying plasma membrane (Seebeck *et al*., 1990). Analysis of optical sections through ConA–TRITC surface-stained cells expressing the GFP–DPMS chimera showed that the DPMS tubule is closely associated with the plasma membrane at the anterior and posterior ends of the cell, as indicated by the overlap of green and red fluorescence in Figure 2e–i. There was rarely overlap in these markers in the middle of the cells. These subpellicular microtubules are resistant to depolymerization by low temperature or standard microtubule depolymerizing compounds such as colchicine or nocodazole, but can be disrupted by phenothiazine derivatives such as thioridazine (Seebeck and Gehr, 1983; Seebeck *et al*., 1990). When thioridazine (5 μg/ml) was added to logarithmic cultures, fluorescence associated with either the DPMS–GFP chimera (Figure 2j) or BODIPY-Cer (unpublished data) retracted to a fluorescent spot(s) that was variably localized along the length of the cell. This effect was observed in all cells within 30 s, with the retraction being essentially instantaneous in individual cells, suggesting that the tubule is normally under elastic tension. In contrast, thioridazine treatment did not affect the morphology of the nuclear envelop and cortical ER containing the GFP–MDDL chimera (Figure 2k) or the mitochondrion which was stained with MitoTracker Red (unpublished data). Throughout these incubations the cells remained motile and metabolically active (see below) for at least 2 h after addition of the drug. Thus, the affect of thioridazine on the DPMS tubule does not appear to be due the non-specific perturbation of intracellular organelles or a gross change in cellular metabolism.

The above results are consistent with the presence of stable intermolecular interactions between the DPMS tubule and the subpellicular microtubule/plasma membrane complex. This putative association was investigated further in cells that had been hypotonically lysed and mechanically disrupted. When crude microsomes were separated by sucrose sedimentation gradient centrifugation, both endogenous DPMS activity (Figure 3A) and fluorescence from GFP–DPMS (unpublished data) were found to cosediment with the plasma membrane marker, acid phosphatase (Gottlieb and Dwyer, 1981). These markers were clearly separated from the endogenous ER lumenal marker, BiP (Figure 3A). Although a small proportion of BiP also sedimented in lighter fractions (in some but not all experiments), the distribution of this fraction was always distinct from that of the DPMS/plasma membrane markers (Figure 3A). Maximum DPMS activity was also consistently displaced from that of the peptide-specific mannophosphotransferase (pepMPT) which initiates protein *O*-phosphoglycosylation in the Golgi apparatus (Moss *et al*., 1999) (Figure 3A). These results suggest that both endogenous DPMS as well as the DPMS–GFP chimera are localized to a distinct membrane subcompartment and supports the notion that the DPMS tubule is connected to the subpellicular cytoskeleton–plasma membrane complex.

The morphology of the DPMS tubule changes during mitosis

Remarkably, GFP–DPMS became localized exclusively to a region proximal to the flagellar pocket in mitotic cells (see green/red overlap in the lower cell of Figure 2l). Immediately after cytokinesis, a single short tubule containing GFP–DPMS was detected from this region and extended toward the posterior end of the cell (Figure 2l, upper cell). The same pattern was observed when cells were stained with BODIPY-C_5-Cer (unpublished data). In contrast, ER membranes containing GFP–MDDL remained intact during mitosis, with both the nuclear envelope and the cortical ER being distributed equally between the two daughter cells (Figure 2m). Similarly, the mitochondrion also remained intact during cell division (unpublished data).

The DPMS tubule may be connected with the Golgi apparatus and closely associated with the mitochondrion

As the anterior end of the DPMS tubule appeared to colocalize with the Golgi apparatus near the flagellar pocket, we investigated the possibility that these organelles

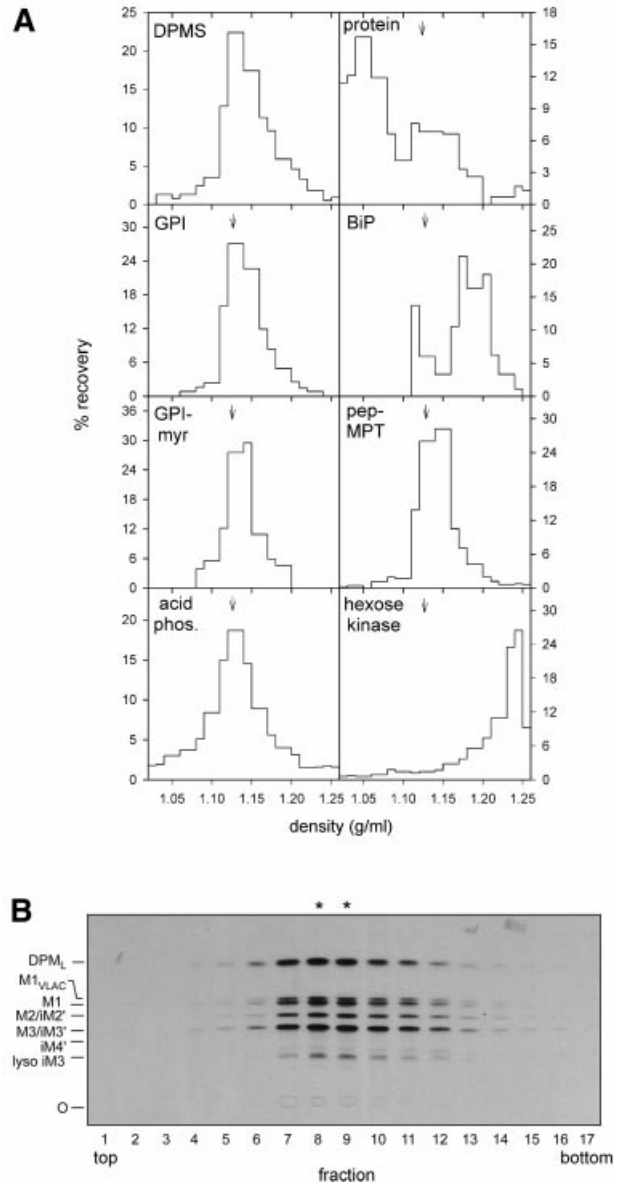

Fig. 3. Localization of DPMS with other cellular markers by subcellular fractionation. Distribution profiles of *L.mexicana* promastigote post-nuclear lysates after isopycnic centrifugation on a linear sucrose gradient (**A**). DPMS (tubule), pep-MPT (Golgi apparatus), acid phosphatase (plasma membrane and flagellar pocket) and hexose kinase (glycosomes) were assayed enzymatically. BiP (ER) was quantified by Western blotting, ECL detection of BiP and densitometry. GPI biosynthesis (GPI) was detected by incubating fractions with GDP–[^{3}H]Man, which is incorporated into endogenous DPM. Myristoylation of GPI species (GPI-myr) was measured by incubating fractions with [^{3}H]myristic acid, ATP and CoA (Ralton and McConville, 1998). The sedimentation position of maximum DPMS activity is indicated by an arrow in the profiles of other markers. (**B**) HPTLC analysis of GPI intermediates generated by sucrose density fractions incubated with GDP–[^{3}H]Man. A short chase with GDP–Man was used to measure the synthesis of both endogenous DPM (DPM$_L$) and GPI intermediates. Maximum DPM and GPI biosynthesis occurred at a density of 1.12–1.15 g/ml (asterisked fractions). The structures of the GPI intermediates are shown in Table I.

may be connected. Initial attempts to express a GFP–chimera containing the *Leishmania donovani* Golgi glycosyltransferase, LPG1p (Ha *et al*., 1996) in *L.mexicana* promastigotes did not give useful fluorescence. Therefore, we generated a new marker for the leishmanial Golgi apparatus by expressing a GFP fusion with the C-terminus of the heterologous human Golgi marker, β-1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-T1–GFP) in *L.mexicana* promastigotes. GlcNAc-T1 is a type-II membrane protein which is localized to the *medial/trans*-Golgi cisternae in animal cells (Burke *et al*., 1994). In *L.mexicana* promastigotes, fluorescence was primarily localized to a single spot, proximal to the flagellar pocket, consistent with a Golgi localization (Ha *et al*., 1996), as well as to an anterior–posterior orientated membrane tubule that was indistinguishable from the DPMS tubule (Figure 4a). GlcNAc-T1–GFP fluorescence in the major anterior spot and the tubule were clearly separated when cells were treated with thioridazine (Figure 4b). Furthermore, GlcNAc-T1–GFP fluorescence in the tubule disappeared during mitosis, while the fluorescence in the anterior 'Golgi' region was retained (Figure 4c). The localization of the GlcNAc-T1–GFP to the Golgi apparatus and DPMS tubule was supported by subcellular fractionation experiments which showed that a significant fraction of this chimera cosedimented with endogenous pep-MPT and DPMS (data not shown). However, a major pool (60–70%) of GlcNAc-T1–GFP cosedimented with endogenous BiP, as determined by immunoblotting with an anti-GFP antibody (data not shown). As no fluorescence was observed in either the nuclear membrane or cortical ER (Figure 4a), it is likely that the ER pool of GlcNAc-T1–GFP is non-fluorescent and possibly misfolded. These data suggest that correctly folded GlcNAc-T1–GFP is localized to both the DPMS tubule and Golgi apparatus, suggesting the presence of vesicular transport between these two membrane compartments. We also investigated the relationship between the DPMS tubule and the mitochondrion of these cells. When cells expressing the GFP–DPMS construct were labeled with MitoTracker Red, close associations between subsections of the DPMS tubule and the mitochondrion were observed consistently (Figure 4d and e).

Colocalization of DPMS with GPI-specific biosynthetic enzymes in vitro

DPM is required for all mannose additions in GPI biosynthesis, except for the α1-3-linked mannose residue in the LPG anchor and hybrid-type GIPLs (Menon *et al*., 1990; Ilgoutz *et al*., 1999). A functional association between DPMS and the DPM-dependent GPI-specific mannosyltransferases was evident when microsomal fractions from the sucrose gradient were pulse–chase labeled with GDP–[^{3}H]Man (Figure 3A). Label was initially incorporated into [^{3}H]DPM and subsequently chased into GPI intermediates with the structures Man$_1$GlcN-PI, Man$_2$GlcN-PI and Man$_3$GlcN-PI (Figure 3B; see Table I and Figure 8 for structures). The tight coupling of DPMS and GPI biosynthetic activities over the profile indicated that these microsomes contained DPMS, the GPI-specific mannosyltransferases, dolichol-phosphate and a supply of early GPI precursors such as GlcN-PI. Most of the GPI intermediates synthesized *in vitro* contained 1-*O*-alkyl(C18:0)-2-acyl-(C18:0)-glycerol lipids and probably represent early intermediates in GIPL biosynthesis (Table I). However, the upper band in the Man$_1$GlcN-PI doublet, M1$_{VLAC}$, contained a 1-*O*-alkyl(C24:0/C26:0)-2-acylglycerol lipid and

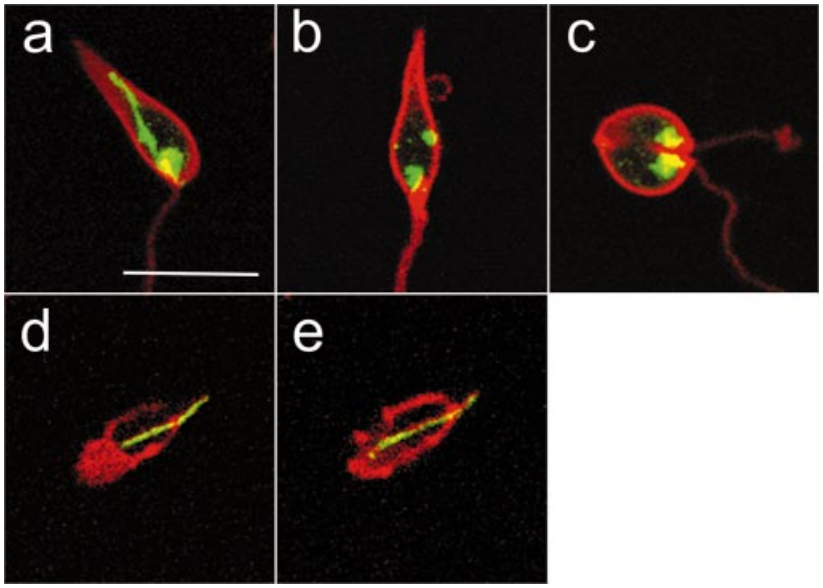

Fig. 4. The DPMS tubule is closely associated with the Golgi apparatus and the mitochondrion. *Leishmania mexicana* promastigotes expressing the GFP chimera containing human *medial*-Golgi marker, GlcNAc-T1 (green) were surface-stained with TRITC-ConA (red) and analyzed by confocal microscopy before (**a**) and after (**b**) treatment with 15 μg/ml thioridazine. (**c**) Dividing cells expressing GlcNAc-T1–GFP. Promastigotes expressing GFP–DPMS (green) were also labeled with the vital stain, Mitotracker Red (red), which stains the mitochondrial reticulum of these cells [(**d**) and (**e**)]. Two consecutive 0.5 μm optical sections taken through the same cell (of a total of eight) show that sections of the DPMS tubule are closely associated with the reticulum of the single mitochondrion (d, e).

Table I. Structures of [^{3}H]Man-labeled GPI lipids synthesized *in vitro* and *in vivo*

GPI	Pathway	Structure
M1	GIPL	$Man_1GlcN\text{-}PI_{18:0'\text{-}18:0}$
$M1_{VLAC}$	anchor	$Man_1GlcN\text{-}PI_{VLAC\text{-}18:0}$
$iM2_{VLAC}$	LPG anchor	$Man\alpha1\text{-}3Man\text{-}GlcN\text{-}PI_{VLAC\text{-}18:0}$
iM2′	GIPL	$Man\alpha1\text{-}3Man\text{-}GlcN\text{-}PI_{18:0'\text{-}18:0}$
M2	GIPL	$Man\alpha1\text{-}6Man\text{-}GlcN\text{-}PI_{18:0'\text{-}18:0}$
iM2	GIPL	$Man\alpha1\text{-}3Man\text{-}GlcN\text{-}PI_{18:0'\text{-}14:0}$
M3	GIPL	$Man\alpha1\text{-}2Man\alpha1\text{-}6Man\text{-}GlcN\text{-}PI_{18:0'\text{-}18:0}$
iM3′	GIPL	$Man\alpha1\text{-}6[Man\alpha1\text{-}3]\ Man\text{-}GlcN\text{-}PI_{18:0'\text{-}18:0}$
iM3	GIPL	$Man\alpha1\text{-}6[Man\alpha1\text{-}3]\ Man\text{-}GlcN\text{-}PI_{18:0'\text{-}14:0}$
iM4′	GIPL	$Man\alpha1\text{-}2Man\alpha1\text{-}6[Man\alpha1\text{-}3]\ Man\text{-}GlcN\text{-}PI_{18:0'\text{-}18:0}$
iM4	GIPL	$Man\alpha1\text{-}2Man\alpha1\text{-}6[Man\alpha1\text{-}3]\ Man\text{-}GlcN\text{-}PI_{18:0'\text{-}14:0}$
EPM3	protein anchor	EtN-P- $Man\alpha1\text{-}2Man\alpha1\text{-}6Man\text{-}GlcN\text{-}PI$

The structures of the protein anchor, LPG anchor and GIPL precursors have been reported previously in Ralton and McConville (1998). These intermediates are assembled on two classes of alkylacyl-PI which contain C18:0 or very long alkyl chains (VLAC, e.g. C24:0, C26:0) at the *sn*-1 position (denoted by a prime). Following assembly of the glycan headgroups, the fatty acyl chain on the *sn*-2 position (mainly C18:0) is replaced with C14:0/C12:0 acyl chains (Ralton and McConville, 1998). Mature GIPL species which have remodeled lipid moieties are not detected when permeabilized cells/microsomes are labeled with GDP–[^{3}H]Man. The nomenclature for these intermediates is as follows. M1-3 refers to the number of mannose residues in the glycan headgroup. The prefix 'i' indicates the presence of the α1-3-linked mannose branch. EP refers to the presence of an EtN-P substituent in the glycan.

is a putative precursor of the protein and LPG anchors (Ralton and McConville, 1998). Other intermediates in GIPL and protein/LPG anchor biosynthesis were not detected in these experiments because a short chase was used to prevent depletion of [^{3}H]DPM. Under these conditions, intermediates containing the α1-3mannose (α1-3Man) branch are not synthesized, as this residue is only added after extended chases in the presence of 0.1 mM GDP–Man (Ilgoutz *et al*., 1999). However, intermediates with mature glycan headgroups were observed in other experiments when microsomes were extensively chased with unlabeled GDP–Man (unpublished data). We also investigated whether these microsomal fractions contained enzymes involved in the remodeling of the *sn*-2 fatty acid of GIPL intermediates having mature glycan headgroups (Ralton and McConville, 1998). The remodeling reactions occur in the absence of DPM synthesis (Ralton and McConville, 1998) and thus provide a separate measurement of the subcellular localization of GPI intermediates. In the presence of CoA and ATP, incorporation of [^{3}H]myristic acid into mature GIPL species (iM2, iM3, iM4) occurred in the same fractions that contained highest DPMS activity (Figure 4A). Thus, microsomes derived from the DPMS tubule appear to contain all the mannosyltransferases involved in GPI anchor and GIPL biosynthesis as well as the acyl-transferase involved in GPI fatty acid remodeling.

The colocalization of DPMS with other GPI-biosyn-

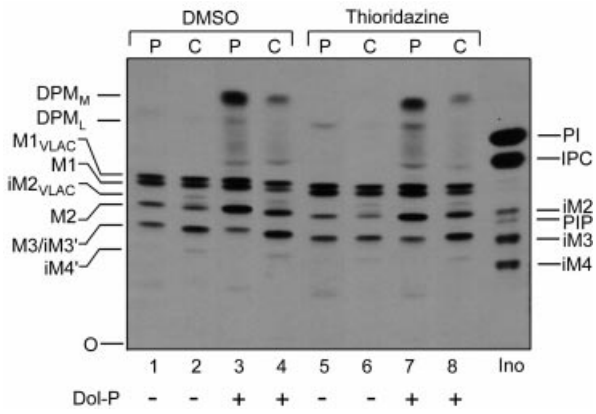

Fig. 5. Functional association of DPMS and GPI-specific mannosyltransferases. *Leishmania mexicana* promastigotes were treated with either 0.3% DMSO (lanes 1–4) or 15 μg/ml thioridazine in DMSO (final concentration 0.3%) (lanes 5–8) before being permeabilized in hypotonic buffer. Crude microsomes were recovered by centrifugation, pulse labeled with GDP–[^{3}H]Man (10 min) in the presence or absence of exogenous dolichol-phosphate, then incubated in the presence of a 1000-fold excess of unlabeled GDP–Man (30 min chase). Aliquots were taken at the end of the pulse (P) and chase (C) periods, and glycolipids analysed by HPTLC. DPM made from endogenous dolichol-phosphate and exogenous mammalian dolichol-phosphate (DPM$_M$) has 11–12 and 16–23 isoprene units, respectively, and can be resolved as two distinct species on HPTLC (Menon *et al*., 1990). The structures of the GPI intermediates synthesized *in vitro* are shown in Table I. Ino refers to [^{3}H]inositol-labeled *L.mexicana* lipids.

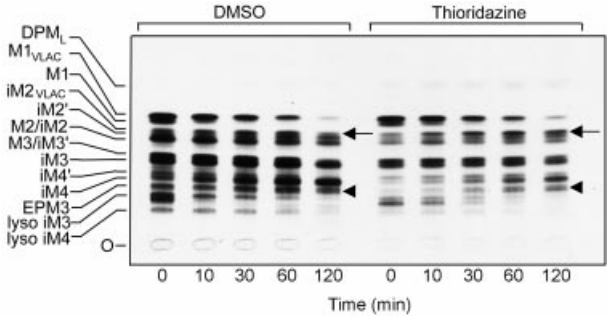

Fig. 6. Effect of thioridazine on GPI biosynthesis *in vivo*. *Leishmania mexicana* promastigotes expressing the GFP–DPMS chimera were incubated in Glc-free RPMI medium containing either 0.3% DMSO (lanes 1–5) or 15 μg/ml thioridazine in DMSO (0.3% final concentration) (lanes 6–10) for 10 min at 27°C and then pulse-labeled with [^{3}H]Man for 10 min. Labeled cells were resuspended in complete medium and aliquots removed at 0 min (lanes 1 and 6), 10 min (lanes 2 and 7), 30 min (lanes 3 and 8), 1 h (lanes 4 and 9) and 2 h (lanes 5 and 10). [^{3}H]Man-labeled GPI was extracted and analyzed by HPTLC in solvent A. [^{3}H]Man was incorporated into a complex profile of GPI intermediates and mature end-products (see Table I for structures). [^{3}H]Man-labeled bands corresponding to the LPG anchor precursor iM2$_{VLAC}$ (arrow) and the protein anchor precursor EPM3$_{VLAC}$ (arrow head) are indicated. Uptake of [^{3}H]Man is partially inhibited in the presence of thioridazine, resulting in a lower rate of incorporation into these GPIs. Note the absence of detectable levels of [^{3}H]DPM in both mock and thioridazine-treated cells, indicating the rapid turnover of newly synthesized DPM.

thetic enzymes was also suggested from *in vitro* labeling experiments using permeabilized cells. When osmotically permeabilized cells were pulse labeled with GDP–[^{3}H]Man, all the endogenous [^{3}H]DPM synthesized in the pulse was rapidly chased into GPI intermediates having both linear and branched (i.e. modified with α1-3Man) glycan headgroups (Figure 5, lanes 1 and 2). Thus, membranes containing DPMS and endogenous pools of dolichol-phosphate also contain the full complement of GPI-specific mannosyltransferases. Similar results were obtained when microsomes were pulse–chase labeled in the presence of both GDP–[^{3}H]Man and exogenous pig liver dolichol-phosphate. Addition of exogenous dolichol-phosphate resulted in a significant stimulation of [^{3}H]DPM synthesis, with a concomitant increase in the amount of label incorporated into more polar GPI intermediates during the chase [as determined by measuring incorporation into each species with a thin layer chromatography (TLC) scanner] (Figure 5, lanes 3 and 4). Note that the [^{3}H]DPM formed from exogenous pig liver dolichol-phosphate had a faster high performance TLC (HPTLC) mobility than endogenous DPM because of differences in the chain length of mammalian and kinetoplastid dolichols (16–23 versus 11–12 isoprene units, respectively) (Menon *et al*., 1990). The fact that the [^{3}H]DPM synthesized from exogenous dolichol-phosphate is also utilized by GPI-specific mannosyltransferases provided further evidence that all the functionally active DPMS was in the same membrane as the GPI biosynthetic enzymes. These experiments were also performed on cells that had been treated with thioridazine prior to osmotic permeabilization, to test whether the colocalization of DPMS with other GPI biosynthetic enzymes could have occurred during lysis. As the effect of thioridazine on the DPMS tubule is extremely rapid and appears to occur without any redistribution of DPMS–GFP to the bulk ER or of GFP–MDDL to the DPMS tubule, we reasoned that thioridazine pretreatment should effectively trap resident integral membrane proteins within the DPMS tubule. As shown in Figure 5 (lanes 5–8), pretreatment with thioridazine had no effect on the chase kinetics of [^{3}H]DPM made from endogenous and exogenous pools of dolichol-phosphate. These data are consistent with DPMS and the GPI-specific mannosyltransferases being present in the same membrane before the cells were lysed.

The effect of thioridazine on GPI biosynthesis was also investigated in living cells. Although thioridazine appears to partially inhibit uptake of [^{3}H]mannose by promastigotes (Figure 6 and unpublished data), the kinetics of incorporation of this sugar into endogenous DPM and GIPL intermediates was essentially the same in control (DMSO-treated) and thioridazine-treated cells (Figure 6). Significantly, [^{3}H]DPM was barely detected at the beginning of the chase, consistent with the rapid utilization of this precursor in both incubations (Figure 6). The major GPI species synthesized in the presence or absence of drug were mature GIPL species (iM2, iM3 and iM4), providing further evidence that all GPI-specific mannosyltransferases and the fatty acid remodeling enzymes are in the same membrane as DPMS. In contrast, thioridazine treatment resulted in the dramatic accumulation of iM2$_{VLAC}$, the first committed LPG anchor precursor (Figure 6). This species is normally chased into mature LPG over 2 h (Ralton and McConville, 1998), but in thioridazine-treated cells it accumulated to be a major GPI species (Figure 6, compare 120 min time points in control and drug-treated cells). The normal chase kinetics of the putative protein anchor precursor, EPM3, was relatively slow in this cell line because it is synthesized in excess of requirements for protein anchoring (unpublished data). However, this

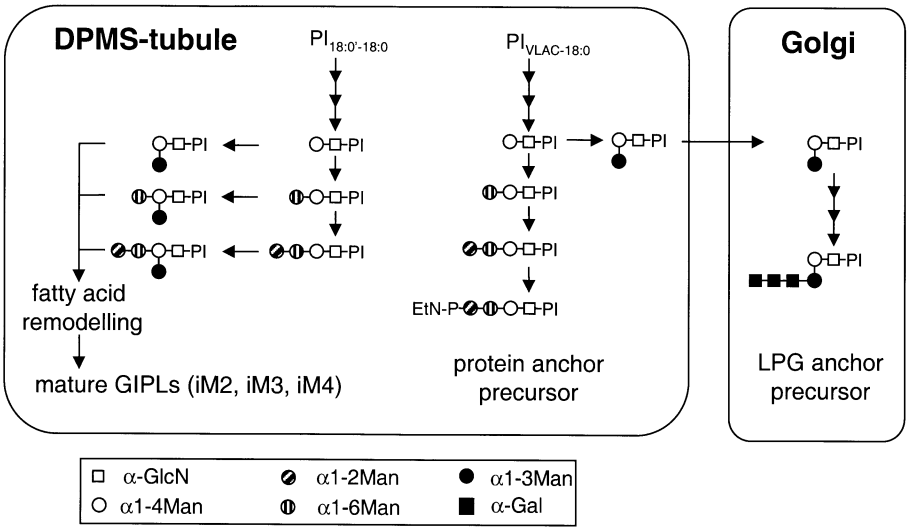

Fig. 7. Summary of GPI biosynthetic reactions which are thought to occur in the DPMS tubule. Based on subcellular fractionation studies and and labeling experiments performed in the presence or absence of a microtubule-disrupting drug, we propose that the DPMS tubule contains four GPI-specific mannosyltransferases, an ethanolamine-phosphotransferase and fatty acid remodeling enzymes involved in biosynthesis of anchor and free GPIs. Assembly of galactosylated intermediates in LPG anchor biosynthesis is thought to occur in the Golgi apparatus (Ha *et al*., 1996), suggesting that $iM2_{VLAC}$ is transported between these two compartments.

intermediate also accumulated in thioridazine-treated cells compared with the control (Figure 6). Thus, disruption of the DPMS tubule structure with thioridazine appears to prevent the further processing of LPG and protein anchor intermediates, either from being modified with phosphoglycan chains in the Golgi apparatus (Ha *et al*., 1996) or from being transferred to protein in the ER, respectively.

Discussion

DPMS and the GPI-specific glycosyltransferases are considered to be markers for the ER, based on both subcellular fractionation and indirect immunolocalization studies in a number of higher and lower eukaryotes (Marriott and Tanner, 1979; Ravoet *et al*., 1981; Preuss *et al*., 1991; Vidugiriene and Menon, 1993, 1994; Prado-Figueroa *et al*., 1994; Takahashi *et al*., 1996; Watanabe *et al*., 1996; Nakamura *et al*., 1997). However, the precise spatial distribution of these enzymes within the ER has not been investigated previously in live cells. In this study we show that DPMS is targeted to a novel membrane compartment in live *L.mexicana* promastigotes (the DPMS tubule), which is probably a discrete subdomain of the ER. In addition, all other DPM-dependent and -independent GPI biosynthetic steps were found to colocalize functionally with DPMS in permeabilized cells, purified microsomes and *in vivo* labeling experiments (Figure 7). Based on these data we propose that the DPMS tubule contains: (i) the three DPM-dependent mannosyltransferases that make the trimannose backbone of the protein anchors and the hybrid-type GIPLs; (ii) the DPM-independent α1-3 mannosyltransferase that specifically modifies LPG anchor and GIPL intermediates; (iii) the ethanolamine-phosphotransferase involved in protein anchor biosynthesis; and (iv) the fatty acid remodeling machinery that exchanges the *sn*-2 C18:0 fatty acid with C14:0/C12:0 fatty acids in GPI intermediates containing mature glycan headgroups (summarized in Figure 8). We did not determine whether the initial steps in GPI biosynthesis (i.e. the formation of GlcNAc-PI and GlcN-PI) were localized in the DPMS tubule. However, there is evidence that GlcNAc-PI is channeled between the de-*N*-acetylase and the mannosyltransferases in both *T.brucei* and *L.major* (Smith *et al*., 1996, 1997), suggesting that these enzymes are also present in the same membrane. Collectively, these analyses suggest that the DPMS tubule is a major biosynthetic compartment for glycolipid biosynthesis in these parasites.

Significantly, perturbation of DPMS tubule structure with thioridazine resulted in the selective accumulation of both the LPG anchor precursor, $iM2_{VLAC}$ and the putative protein anchor precursor, EPM3. The $iM2_{VLAC}$ intermediate is further modified with three galactose residues, the first of which is added by the Golgi-located galactofuranosyltransferase, LPG1p (Huang and Turco, 1993; Ha *et al*., 1996). It is thus possible that perturbation of the DPMS tubule prevents transport of this intermediate from the DPMS tubule to the Golgi apparatus, although we cannot discount the possibility that thioridazine selectively inhibits LPG1p. Similarly, thioridazine may inhibit the transport of the mature protein anchor GPI precursors from the DPMS tubule to ER compartments containing nascent proteins, or alternatively inhibit the transport of nascent proteins to the tubule. Collectively, these data suggest that the DPMS tubule is not a dead-end compartment, but that it may be connected either directly or by vesicular transport to both the bulk ER and the Golgi apparatus (discussed below).

The intracellular organization of the DPMS tubule may be maintained by specific connections with the subpellicular microtubules that underly the plasma membrane and possibly also through associations with other intracellular organelles such as the mitochondrion (Figure 5). Direct connections between the subpellicular microtubules and the DPMS tubule were suggested by the colocalization of sections of this tubule with the plasma membrane by confocal microscopy, subcellular fractionation experiments and the sensitivity of this tubule to the cytoskeletal disrupting agent, thioridazine. Addition of

thioridazine resulted in the rapid retraction of the DPMS tubule, while other organelles (e.g. the bulk ER, Golgi apparatus, mitochondrion) were unaffected. The speed of this retraction (<1 s) suggests that the tubule is under elastic tension in the cell, and that relatively few connections with the cytoskeleton are involved or that multiple connections are broken simultaneously. The molecular basis for the action of thioridazine on these subpellicular microtubules is unknown, but may involve a putative 60 kDa thioridazine-binding protein which is associated with microtubules (Stieger and Seebeck, 1986). This protein could either stabilize microtubule structure or act as a specific linker between the cytoskeleton and the DPMS tubule. Interestingly, cortical ER elements containing the GFP–MDDL construct also appear to be closely associated with the plasma membrane in living cells, although these connections are apparently not sensitive to thioridazine or retained after cell lysis (Figure 4). Stable connections between elements of the smooth ER and the subpellicular microtubules have previously been detected by electron microscopy in both *Leishmania* (Pimenta *et al*., 1991) and *T.brucei* (Vickerman *et al*., 1969; Sherwin and Gull, 1989). In *T.brucei* bloodstream forms, smooth ER profiles are specifically associated with a set of four microtubules which originate from the basal body, a microtubule organization centre at the base of the flagellum, and extend along the anterior–posterior axis of the cell (Sherwin and Gull, 1989). Microtubules in this quartet have the opposite polarity to the other subpellicular microtubules (plus end directed toward the posterior of the cell), are comparatively resistant to low temperature/high salt solubilization, and contain a novel form of γ-tubulin along their length (Robinson *et al*., 1995; Scott *et al*., 1997). As the DPMS tubule also appears to be anchored to the anterior of the cell and extend along the anterior–posterior axis of the cell, it is possible that these microtubules are required for the biogenesis and maintenance of the DPMS tubule.

Our studies provide another example of functional heterogeneity in the ER (Sita and Meldolesi, 1992; Staehlin, 1997). Moreover, they show that compartmentalization of the ER beyond the formation of the classical ER subdomains (i.e. the rough ER, smooth ER and nuclear envelope) occurs even in these evolutionarily primitive eukaryotes. Interestingly, recent evidence suggests that some steps in GPI biosynthesis may also be compartmentalized in the ER of animal cells (Watanabe *et al*., 1996). In particular, biochemical analysis of subcellular fractions obtained from mouse thymoma cell lines suggest that the second step (conversion of GlcNAc-PI to GlcN-PI) and subsequent steps in protein anchor biosynthesis are segregated into ER subdomains which are tightly associated with mitochondria (J.Vidugiriene, D.K.Sharma, T.K.Smith, N.A.Baumann and A.K.Menon, manuscript submitted). Sections of the DPMS tubule were also closely associated with the single mitochondrial reticulum in *L.mexicana* promastigotes (Figure 5), raising the possibility that the DPMS tubule may be functionally related to the mitochondria-associated ER membranes of animal cells. The reason why GPI biosynthetic enzymes are compartmentalized in the DPMS tubule is unknown. The specific lipid precursors PI, dolichol-phosphate and phosphatidylethanolamine may be concentrated in this subdomain, which is likely to have a unique lipid composition based on the remarkably specific accumulation of BODIPY-C_5-Cer in the DPMS tubule. Indeed, the sequestration of specific alkylacyl-PI molecular species (which accounts for <20% of the total PI pool; Ralton and McConville, 1998) in this domain may explain why these lipids are selectively incorporated into these GPIs. However, it remains to be determined how the distinct alkylacyl-PI lipid compositions of the anchor GPIs and the GIPLs are achieved.

In addition to being a functionally important compartment in glycolipid biosynthesis, our data also suggest that the DPMS tubule is closely associated with the anteriorly located Golgi apparatus. First, the anterior end of the tubule always terminates in a region of the cell juxtaposed to the flagellar pocket which is also the site of the Golgi apparatus (Pimenta *et al*., 1991; Cappai *et al*., 1993; Ha *et al*., 1996; Ma *et al*., 1997). An association with one or more organelles in this region is further suggested by the restricted localization of the two DPMS tubule markers (GFP–DPMS, BODIPY-C_5-Cer) to the flagellar pocket/Golgi region during mitosis. Secondly, the colocalization of the heterologous Golgi marker, GFP–GlcNAc-T1, in both the Golgi apparatus and the DPMS tubule strongly suggests that at least some proteins are transported between these two organelles and therefore that the tubule is not a dead-end compartment. At present, we cannot discount the possibility that the DPMS tubule is a subcompartment of the Golgi apparatus, although previous studies using both fluorescence and electron micrcroscopy suggest that several endogenous leishmanial Golgi markers, including a Rab1 homologue which should be associated with the *cis*-most Golgi compartment, are restricted to the anteriorly located tubular-vesicular Golgi complex and are not found in membranes reminiscent of the DPMS tubule (Cappai *et al*., 1993; Ha *et al*., 1996; Ma *et al*., 1997). It is thus likely that the partial localization of GlcNAc-T1 in the DPMS tubule represents a degree of mislocalization of this chimera. As the localization of GlcNAc-T1 in the *medial*-Golgi cisternae of animal cells is thought to involve the formation of homo- or hetero-oligomers with other Golgi proteins and/or lipid-based sorting mechanisms (Burke *et al*., 1994; Colley, 1997), some mislocalization of this construct in *Leishmania* might be expected. Thirdly, as mentioned above, the accumulation of the LPG anchor precursor in thioridazine-treated cells is consistent with the notion that some GPI intermediates synthesized in the DPMS tubule are subsequently transported to the Golgi (Figure 8). Based on these properties, we suggest that the DPMS tubule corresponds to a stable transitional ER, where proteins and lipids are packaged into vesicles for transport to the Golgi apparatus. This notion is supported by the absence of detectable levels of ER luminal markers, GFP–MDDL and endogenous BiP, in the DPMS tubule, consistent with this organelle being a pre-Golgi compartment from which soluble ER resident proteins are either excluded or rapidly retrieved back to the bulk ER (Vertel *et al*., 1989; Banykh and Balch, 1997; Hobman *et al*., 1998). In other eukaryotes, transitional ER subdomains can either be short-lived structures which are dispersed throughout the ER (e.g. in *Saccharomyces cerevisiae* and many plant and vertebrate cells) or relatively stable structures which are juxtaposed with the *cis*-Golgi network (Orci *et al*., 1991; Bannykh and Balch, 1997; Presley

et al., 1997; Staehelin, 1997). The latter situation exists in some lower eukaryotes (e.g. *Pichia pastoris*) and higher eukaryotic cells which are synthesizing large quantities of secretory proteins (Orci *et al*., 1991; Staehelin, 1997; Rossanese *et al*., 1999). The presence of an amplified transitional ER in *L.mexicana* promastigotes may reflect the high secretory capacity of this developmental stage. Alternatively, an expanded transitional ER could replace long distance vesicular transport (i.e. from ER at the posterior end of the cell), which is likely to be inefficient in *Leishmania* and other kinetoplastid parasites due to the absence of transcellular microtubules along which transport vesicles could be carried (Seebeck *et al*., 1990; Bloom and Goldstein, 1998). The availability of specific markers for the DPMS tubule will facilitate future studies on the precise nature and function of this compartment. Finally, our studies suggest that perturbation of the DPMS tubule is likely to have profound effects on the synthesis and intracellular transport of GPI-anchored proteins and LPG, as well as other plasma membrane components (K.A.Mullin and M.J.McConville, unpublished data). These data provide an additional basis for the cytotoxicity of phenothiozines to *Leishmania* and other kinetoplastid parasites (Zilberstein and Dwyer, 1984) and suggest that proteins involved in maintaining the DPMS tubule are potential targets for the development of new anti-parasite compounds.

Materials and methods

Parasite culture and transformation

Leishmania mexicana (strain MNYC/BZ/62/M379) promastigotes were cultivated at 27°C in RPMI medium supplemented with 10% fetal bovine serum (FBS). The transformation protocol was essentially the same as described by Kapler *et al*. (1990). Cells were grown to late log density (1×10^7 cells/ml), harvested by centrifugation, washed once in electroporation buffer (21 mM HEPES–NaOH pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 6 mM glucose) and then resuspended in electroporation buffer at a density of 1×10^8 cell/ml. Transformations were performed using a Bio-Rad Gene Pulser. Cells (0.4 ml) were added to an electroporation cuvette and held on ice for 10 min. Plasmid DNA (30 μg) was added and the cuvette pulsed at a voltage of 2250 V/cm and a capacitance of 500 μF. Cells were held on ice for 10 min and then transferred to 50 ml RPMI containing 10% FBS. After 18 h, transformants were selected by dilution of the culture in fresh medium containing G418 (30 μg/ml).

DNA constructs

All cloning was performed in *E.coli* XL1-Blue-MRF′ (Stratagene). The following synthetic oligonucleotides were used to PCR-amplify the various DNA constructs used in this study (nucleotides in bold denote a sequence which was not complementary to template DNA, but added to maintain reading frames and/or to incorporate restriction sites for cloning; start codons are underlined and stop codons are double underlined): primer 1, GACTGGATCCCATGCAGTACTCCATTATCG, 5′ forward primer for DPMS with a *Bam*HI site; primer 2, GACTGAATTCTAGACCTAGAAGAGGGAATGGTAGAG, 3′ reverse primer for DPMS with an *Xba*I site; primer 3, ATCGCCCGGGATGAGCAAGGGCGAGGAG, 5′ forward primer for GFP with a *Sma*I site; primer 4, ATGCGGATCCTTGTACAGCTCGTCCATGC, 3′ reverse primer for GFP with a *Bam*HI site; primer 5, GTACTCTAGAGTGAGCAAGGGCGAGGAG, 5′ forward primer for C-terminal addition of MDDL to GFP with an *Xba*I restriction site; primer 6, GTATCCGCGGTTACAGATCGTCCATCTTGTACAGCTCGTCCATGC, 3′ reverse primer for C-terminal MDDL, incorporating a *Ksp*I site; primer 7, GGGATGTCGAGGATGTGGCTGAC, 5′ forward primer for N-terminal signal sequence of *T.brucei* BiP; primer 8, GTACTCTAGAGTATGTTGTGCCGAGGTCGATG, 3′ reverse primer for N-terminal signal sequence of *T.brucei* BiP with an *Xba*I site; primer 9, ACGCGGATCCATGCTGAAGAAGCAGTCTGC, 5′ forward primer for human GlcNAc-T1 containing a *Bam*HI site; primer 10, ATCATTCCAGCTAGGATCATAGCC, 3′ reverse primer for human GlcNAc-T1 containing half the *Eco*RV site.

For assembly of pGFP-DPMS (encoding a GFP–DPMS fusion protein) (Figure 1), the DPMS gene was amplified by PCR from a leishmanial DPMS genomic clone (Ilgoutz *et al*., 1999) using primers 1 and 2, then cloned into *Bam*HI–*Xba*I-cut pX (LeBowitz *et al*., 1990) using the restriction sites introduced into the PCR amplicon. The gene which encodes GFP was amplified from pXG-′GFP+ (Ha *et al*., 1996) using primers 3 and 4, digested with *Sma*I and *Bam*HI, then cloned upstream of, and in frame with, the DPMS gene in pX. pGFP-MDDL encodes a recombinant GFP with an N-terminal signal sequence and the C-terminal MDDL tetrapeptide, which functions as an ER retention signal in *T.brucei* (Bangs *et al*., 1993) (Figure 1). The GFP gene fused to the MDDL retention signal was generated as a single amplicon using the primers 5 and 6 and pXG-′GFP+ (Ha *et al*., 1996) as template and cloned into pX cut with *Xba*I–*Ksp*I. An amplicon encoding the N-terminal signal sequence was amplified directly from genomic DNA prepared from *T.brucei* using primers 7 and 8. This was digested with *Xba*I and cloned upstream of, and in frame with, the GFP/retention signal chimera, thus creating a gene which encodes a signal sequence/GFP/MDDL fusion protein (GFP–MDDL). The plasmid pGFP contains a gene which encodes GFP with no signal sequences (Figure 1), cloned into pX. The GFP gene was excised from pEGFP-C1 (Clontech, USA) using the restriction endonucleases *Eco*47III and *Bcl*I, then cloned into pX digested with *Sma*I–*Bam*H1.The plasmid pGlcNAc-T1–GFP encodes GFP fused to the C-terminus of human β-1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-T1–GFP; Figure 1). The GlcNAc-T1 gene was amplified from the template pICneo (Burke *et al*., 1994) and directionally cloned into the GFP-containing vector pXG-′GFP+ digested with *Bam*HI and *Eco*RV (Ha *et al*., 1996). Plasmid DNA used in transformations was purified from *E.coli* cultures using anion exchange columns (Qiagen) and resuspended in 10 mM Tris–HCl (pH 8.0).

Labeling of the cell surface and intracellular organelles of live cells with vital stains

The cell surface and flagellar pocket of live *L.mexicana* promastigotes were visualized with ConA–TRITC (Sigma). Cells were gently pelleted by centrifugation (5000 *g*, 30 s) and resuspended in phosphate-buffered saline (PBS) containing 5 μl/ml ConA–TRITC and 1% BSA. After incubation for 3 min at 27°C, cells were gently pelleted and resuspended in PBS containing 1% BSA. The mitochondrion was labeled with the vital stain MitoTracker Red CMXRos (Molecular Probes Inc.) which was added to medium from a 1 mM stock solution in dimethylsulfoxide (DMSO) to a final concentration of 1 nM. After incubation for 10 min at 27°C, cells were gently pelleted and resuspended in fresh medium.

Labeling of cells with BODIPY-C_5-Cer [*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosine; Molecular Probes Inc.] was performed as follows: *L.mexicana* promastigotes (5×10^7 cells) were pelleted by centrifugation, and incubated in RPMI medium containing 2 mg/ml BSA for 15 min at 27°C. The suspension was supplemented with BODIPY-C_5-ceramide bound to defatted BSA (from a stock solution containing 1 μg BODIPY-ceramide and 6 mg BSA/ml) to give a final concentration of 0.1 μg BODIPY-ceramide/ml. After 10 min at 27°C, cells were gently pelleted by centrifugation and resuspended in RPMI medium containing 2 mg/ml defatted BSA.

Confocal microscopy

Cells were immobilized for fluorescence microscopy by mounting under poly-L-lysine coated coverslips. Samples were viewed with a Bio-Rad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan II microscope with a krypton/argon laser. Differential visualization of the fluorophore was achieved using a 488 nm excitation filter and 522/535 nm emission filter for green fluorescence, and a 568 nm excitation filter and 605/632 nm emission filter for red fluorescence. Samples were scanned to simultaneously collect light emitted as red or green fluorescence using a 100× objective (NA 1.4) and 3× zoom. Transmitted light images were collected at the same time as fluorescent images. Images of 512×512 pixels were obtained using Bio-Rad Lasersharp software and processed using Adobe Photoshop software.

Subcellular fractionation

Leishmania mexicana promastigotes (8×10^8) were harvested by centrifugation (1000 *g*, 10 min) and washed in PBS. All steps were performed at 4°C. Cells were washed in hypotonic buffer [2 mM EGTA, 2 mM DTT, 2 mM leupeptin, 0.2 mM TLCK, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], recovered by centrifugation and resuspended in

hypotonic buffer at a concentration of 2×10^8 cells/ml (5 min, 4°C). Cell lysis was achieved by expulsion (×10) of the suspension through a 27 gauge needle. The cell lysate was made isotonic by addition of 4× assay buffer A (50 mM HEPES–NaOH pH 7.4, 0.25 M sucrose, 1 mM ATP, 1 mM EGTA, 2 mM DTT, 2 mM leupeptin, 0.2 mM TLCK, 0.1 mM PMSF), centrifuged at 3000 *g* for 10 min to remove cell debris and nuclei, and the supernatant layered on top of a linear sucrose gradient (density 1.05–1.27 g/ml). The gradient was prepared by layering ten 0.8-ml fractions (0.25–2 M sucrose in 25 mM HEPES–NaOH, pH 7.4) over a sucrose cushion (2.5 M) in Ultraclear Centrifuge tubes (Beckman) followed by centrifugation at 218 000 *g* for 1 h. Organelles in the 3000 *g* supernatant were fractionated by centrifugation at 218 000 *g* for 6 h at 4°C in a Beckman L-80 Ultracentrifuge using a SW41 rotor. Fractions (0.5 ml) were collected from the bottom of the tube and densities calculated by measuring the refractive index. DPMS activities were measured using a modification of the procedure of Prado-Figueroa (1994). Aliquots of the gradient fractions were diluted to give a final buffer concentration of 50 mM HEPES–NaOH pH 7.4, 2 mM EGTA, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 1 mM ATP, 0.2 mM TLCK, 2 mM leupeptin, 0.1 mM PMSF, 0.4% Triton X-100, 75 mM dolichol-phosphate (C_{40}-C_{65}) and 300 mM GDP–[^{3}H]Man (0.3 μCi) and incubated at 15°C for 40 min. [^{3}H]DPM was recovered in the upper organic phase after addition of 200 μl water-saturated 1-butanol and biphasic partitioning and quantitated by scintillation counting. GPI biosynthesis in microsomal fractions was measured by diluting an aliquot of the gradient in 5× buffer A and incubation with 1 mM GDP–[^{3}H]Man (0.3 μCi) for 10 min at 27°C (Ralton and McConville, 1998). The reaction was stopped by the addition of 2 vol. water-saturated 1-butanol and the [^{3}H]GPIs recovered by two-phase partitioning as described for the DPMS assay. [^{3}H]GPIs were resolved by chromatography on aluminium-backed silica gel 60 HPTLC sheets (MERCK) developed in solvent A, and detected by fluorography after spraying the sheets with EN^{3}HANCE™ (DuPont) and exposing them to BioMax film (Kodak) (Ralton and McConville, 1998). GPI myristoylation was measured using the same buffer (but minus [^{3}H]GDP–Man) containing 0.5 μCi [9,10-^{3}H]myristic acid (11.2 Ci/mmol) and CoA (0.2 mM) (Ralton and McConville, 1998). The microsomes were incubated for 1 h at 20°C before extraction of labeled GPIs and processing as described above. The peptide-specific mannose-phosphotransferase (pep-MPT), which adds Manα1-PO_4 to serine residues in several secreted glycoproteins and proteoglycans, is a marker for the Golgi apparatus in *L.mexicana* (Moss *et al*., 1999). Pep-MPT activity was measured in 50 mM HEPES–NaOH pH 7.4, containing 0.4% Triton X-100, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2.5 mM dithiothreitol, 1 mM ATP, 0.2 mM TLCK and 2 mM leupeptin, using a synthetic 19 amino acid peptide acceptor (sapp-1) and 1 mM GDP–[^{3}H]Man as donor, as described previously (Moss *et al*., 1999). The lumenal ER marker, BiP, was detected by immunoblotting using an antibody raised against the *T.brucei* BiP (Bangs *et al*., 1993). This antibody recognized a single 70 kDa protein band when *L.mexicana* lysates were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), suggesting that it specifically recognizes the *L.mexicana* BiP homologue. Proteins were precipitated from gradient fractions (Wessel and Flugge, 1984) and separated using 12% SDS–PAGE prior to electrophoretic transfer to nitrocellulose using a Trans-Blot (Bio-Rad) apparatus. For detection of the BiP, the nitrocellulose was blocked with 0.05% Tween-20 in Tris-buffered saline (1 h, 25°C), incubated with rabbit anti-BiP antibody (1 h, 25°C) (Bangs *et al*., 1993), washed with 0.05% Tween-20 in Tris-buffered saline, then probed with anti-rabbit horse radish peroxidase conjugated antibody (1:10 000 dilution; Silenus) in 0.05% Tween-20 in Tris-buffered saline containing 1% powdered skimmed milk (1 h, 25°C). After washing, specific binding was detected using the ECL Western Detection System (Amersham). Acid phosphatase (marker for plasma membrane and lysosomes) and hexose kinase (marker for glycosomes) were measured using published procedures (Bergmeyer *et al*., 1974; Gottlieb and Dwyer, 1981).

Assay for GPI biosynthesis in vivo and in permeabilized cells

For analysis of GPI biosynthesis *in vivo*, *L.mexicana* promastigotes were suspended at 2×10^8 cells/ml in glucose-free RPMI medium containing 1% BSA, 1 μg/ml tunicamycin and either 0.3% DMSO or 15 μg/ml thioridazine (from a stock solution in DMSO, diluted to give a final DMSO concentration of 0.3%). The promastigotes were incubated at 27°C for 30 min before the addition of [^{3}H]Man (100 μCi, 17 Ci/mmol). After a 5 min pulse, cells were pelleted by centrifugation (5000 *g*, 30 s) and resuspended at 2×10^8 cells/ml in complete RPMI medium containing 10% FBS. Aliquots (5×10^6 cell equivalents) were harvested by centrifugation at 10, 30, 60 and 120 min, and extracted in chloroform/methanol/water (1:2:0.8 v/v, 380 μl) for 2 h at 25°C. Insoluble material was removed by centrifugation (14 000 *g*, 2 min) and the supernatants dried under N_2. [^{3}H]GPIs were recovered biphasic partitioning in 1-butanol (200 μl) and water (100 μl) as described above, resuspended in 40% 1-propanol (10 μl) and analyzed on aluminium-backed Silica-Gel-60 HPTLC sheets developed in solvent A. HPTLC sheets were sprayed with EN^{3}HANCE™ (DuPont) and exposed to BioMax film (Kodak).

Untreated or thioridazine-treated promastigotes (15 μg/ml, 5 min, 27°C) were permeabilized by hypotonic lysis as described above, without passage through the narrow bore needle. Permeabilized cells were recovered by centrifugation (6000 *g*, 5 min), then resuspended in buffer A (4×10^8 cell equivalents/ml). This suspension was supplemented with pig liver dolichol-phosphate (0.4 mg/ml, added from a 4 mg/ml stock solution in 0.2% Triton X-100) or 0.02% Triton X-100 as control. Labeling was initiated by adding GDP–[^{3}H]Man (0.5 μCi) and incubation of the permeabilized cells for 10 min at 27°C. Unlabeled GDP–Man (1 mM) and the incubation continued for another 30 min. [^{3}H]GPIs were extracted in chloroform/methanol/water (1:2:0.8 v/v), recovered by 1-butanol/water phase partitioning and analyzed by HPTLC.

Acknowledgements

We thank Drs Paul Gleeson and Julie Ralton for critical reading of the manuscript, Dr Paul Gleeson for providing the plasmid containing human GlcNAc-T1, Dr James Bangs for the anti-BiP antibody, and Professor Steve Beverley and Dr Haiyan Xu for pX-NEO vectors and the GFP construct. This work was supported by grants from the Australian National Health and Medical Research Council and Wellcome Trust. M.J.M. is a Wellcome Trust Senior Research Fellow.

References

Bangs,J.D., Uyetake,L., Brickman,M.J., Balber,A.E. and Boothroyd,J.C. (1993) Molecular cloning and cellular localization of a BiP homologue in *Trypanosoma brucei*. Divergent ER retention signals in a lower eukaryote. *J. Cell Sci*., **105**, 1101–1113.

Bannykh,S.I. and Balch,W.E. (1997) Membrane dynamics at the endoplasmic reticulum–Golgi interface. *J. Cell Biol*., **138**, 1–4.

Bergmeyer,H.U., Gawehn,K. and Grassl,M. (1974) Enzymes as biochemical reagents. In Bergmeyer,H.U. (ed.), *Methods in Enzymatic Analysis*. Verlag Chemie, Weinheim, pp. 425–522.

Bloom,G.S. and Goldstein,L.S.B. (1998) Cruising along microtubule highways: how membranes move along the secretory pathway. *J. Cell Biol*., **140**, 1277–1280.

Burke,J., Pettit,J.M., Humphris,D. and Gleeson,P.A. (1994) *Medial* Golgi retention of *N*-acetylglucosaminyltransferase I. Contribution from all domains of the enzyme. *J. Biol. Chem*., **269**, 12049–12059.

Cappai,R., Osborn,A.H., Gleeson,P.A. and Handman,E. (1993) Cloning and characterization of a Golgi-associated GTP-binding protein homologue from *Leishmania major*. *Mol. Biochem. Parasitol*., **62**, 73–82.

Colley,K.J. (1997) Golgi localization of glycosyltransferases: more questions than answers. *Glycobiology*, **7**, 1–13.

Ferguson,M.A.J. (1997) The surface glycoconjugates of Trypanosomatid parasites. *Philos. Trans. R. Soc. Lond. B*, **352**, 1295–1302.

Gottlieb,M. and Dwyer,D.M. (1981) *Leishmania donovani*: surface membrane acid phosphatase activity of promastigotes. *Exp. Parasitol*., **52**, 117–128.

Ha,S.D., Schwarz,J.K., Turco,S.J. and Beverley,S.M. (1996) Use of green fluorescent protein as a marker in transfected *Leishmania*. *Mol. Biochem. Parasitol*., **77**, 57–64.

Hirshberg,C.B. and Schneider,M.D. (1987) Topology of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu. Rev. Biochem*., **56**, 63–87.

Hobman,T.C., Zhao,B., Chan,H. and Farquhar,M.G. (1998) Immunoisolation and characterization of a subdomain of the endoplasmic reticulum that concentrates protein involved in COPII vesicle biogenesis. *Mol. Biol. Cell*, **9**, 1265–1278.

Huang,C.C. and Turco,S.J. (1993) Defective galactofuranose addition in lipophosphoglycan biosynthesis in a mutant of *Leishmania donovani*. *J. Biol. Chem*., **268**, 24060–24066.

Ilgoutz,S.C., Zawadzki,J.L., Ralton,J.E. and McConville,M.J. (1999) Evidence that free GPI glycolipids are essential from growth of *L.mexicana*. *EMBO J*., **18**, 2746–2755.

Kapler,G.M., Coburn,C.M. and Beverley,S.M. (1990) Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase

region sufficient for extrachromosomal replication and expression. *Mol. Cell. Biol.*, **10**, 1084–1094.
LeBowitz,J.H., Coburn,C.M., McMahon-Pratt,D. and Beverley,S.M. (1990) Development of a stable *Leishmania* expression vector and application to the study of parasite surface antigen genes. *Proc. Natl Acad. Sci. USA*, **87**, 9736–9740.
McConville,M.J. and Blackwell,J.M. (1991) Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*: characterization of the promastigote and amastigote glycolipids. *J. Biol. Chem.*, **266**, 15170–15179.
McConville,M.J. and Ferguson,M.A.J. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.*, **294**, 305–324.
McConville,M.J., Collidge,T.A.C., Ferguson,M.A.J. and Schneider,P. (1993) The glycoinositol phospholipids of *Leishmania mexicana*. Evidence for the presence of three distinct pathways of glycolipid biosynthesis. *J. Biol. Chem.*, **268**, 15595–15604.
Marriott,M. and Tanner,W. (1979) Localization of dolichyl phosphate- and pyrophosphate-dependent glycosyl transfer reactions in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **139**, 566–572.
Menon,A.K., Mayor,S. and Schwarz,R.T (1990) Biosynthesis of glycosyl-phosphatidylinositol lipids in *Trypanosoma brucei*: involvement of mannosyl-phosphoryl-dolichol as the mannose donor. *EMBO J.*, **9**, 4249–4258.
Moss,J.M., Reid,G.E., Mullin,K.A., Zawadzki,J.L., Simpson,R.J. and McConville,M.J. (1999) Characterization of a novel GDP–mannose:serine-protein mannose-1-phosphotransferase from *Leishmania mexicana*. *J. Biol. Chem.*, **274**, 6678–6688.
Nakamura,N., Inoue,N., Watanabe,R., Takahashi,M., Takeda,J., Stevens,V.L. and Kinoshita,T. (1997) Expression cloning of the human PIG-L, a candidate *N*-acetylglucosaminylphosphatidylinositol deacetylase. *J. Biol. Chem.*, **272**, 15834–15840.
Orci,L., Ravazzola,M., Meda,P., Holcomb,C., Moore,H.-P., Hicker,L. and Schekman,R. (1991) Mammalian Sec22p homologue is restricted to the endoplasmic reticulum transitional cytoplasm. *Proc. Natl Acad. Sci. USA*, **88**, 8611–8615.
Orlean,P. (1990) Dolichol phosphate mannose synthase is required *in vivo* for glycosyl-phosphatidylinositol membrane anchoring, *O* mannosylation and *N* glycosylation of protein in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **10**, 5796–5805.
Overath,P., Stierhof,Y.-D. and Weise,M. (1997) Endocytosis and secretion in trypanosomatid parasites—tumultuous traffic in a pocket. *Trends Cell Biol.*, **7**, 27–33.
Pagano,R.E., Sepanski,M.A. and Martin,O.C. (1989) Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a *trans*-Golgi marker for both light and electron microscopy. *J. Cell Biol.*, **109**, 2067–2079.
Pimenta,P.F.P., Saraiva,E.M.B. and Sacks,D.L. (1991) The comparative fine structure and surface glycoconjugate expression of three life cycle stages of *Leishmania major*. *Exp. Parasitol.*, **72**, 191–204.
Prado-Figueroa,M., Raper,J. and Opperdoes,F.R. (1994) Possible localization of dolichol dependent mannosyltransferase of *Trypanosoma brucei* to the rough endoplasmic reticulum. *Mol. Biochem. Parasitol.*, **63**, 255–264.
Presley,J.F., Cole,N.B., Schroer,T.A., Hirshberg,K., Zaal,K.J.M. and Lippincott-Schartz,J. (1997) ER–Golgi transport visualized in living cells. *Nature*, **389**, 81–85.
Preuss,D., Mulholland,J., Kaiser,C.A., Orlean,P., Albright,C., Rose,M.D., Robbins,P.W. and Botstein,D. (1991) Structure of the yeast endoplasmic reticulum: localization of the ER protein using immunofluorescence and immunoelectron microscopy. *Yeast*, **7**, 891–911.
Proudfoot,L., Schneider,P., Ferguson,M.A.J. and McConville,M.J. (1995) Biosynthesis of the glycolipid anchor of lipophosphoglycan and the structurally related glycoinositolphospholipids from *Leishmania major*. *Biochem. J.*, **308**, 45–55.
Puoti,A., Desponds,C. and Conzelmann,A. (1991) Biosynthesis of mannosylinositol phosphoceramide in *Saccharomyces cerevisiae* is dependent on genes controlling the flow of secretory vesicles from the endoplasmic reticulum to the Golgi. *J. Cell Biol.*, **113**, 515–525.
Ralton,J.E. and McConville,M.J. (1998) Delineation of three pathways of glycosylphosphatidylinositol biosynthesis in *Leishmania mexicana*. Precursors from different pathways are assembled on distinct pools of phosphatidylinositol and undergo fatty acid remodeling. *J. Biol. Chem.*, **273**, 4245–4257.
Ravoet,A.-M., Amar-Costesec,A., Godelaine,D. and Beaufay,H. (1981) Quantitative assay and subcellular distribution of enzymes acting on dolichyl phosphate in rat liver. *J. Cell Biol.*, **91**, 679–688.
Robinson,D.R., Sherwin,T., Ploubidou,A., Byard,E.H. and Gull,K. (1995) Microtubule polarity and dynamics in the control of organelle positioning, segregation and cytokinesis in the Trypanosome cell cycle. *J. Cell Biol.*, **128**, 1163–1172.
Rossanese,O.W., Soderholm,J., Bevis,B.J., Sears,I.B., O'Conner,J., Williamson,E.K. and Glick,B.S. (1999) Golgi structure correlates with transitional endoplasmic reticulum organization in *Pichia pastoris* and *Saccharomyces cerevisiae*. *J. Cell Biol.*, **145**, 69–81.
Scott,V., Sherwin,T. and Gull,K. (1997) γ-Tubulin in trypanosomes: molecular characterization and localization to multiple and diverse microtubule organizing centres. *J. Cell Sci.*, **110**, 157–168.
Seebeck,T. and Gehr,P. (1983) Trypanocidal action of neuroleptic phenothiazines in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.*, **9**, 197–208.
Seebeck,T., Hemphill,A. and Lawson,D. (1990) The cytoskeleton of Trypanosomes. *Parasitol. Today*, **6**, 49–52.
Sherwin,T. and Gull,K. (1989) The cell division cycle of *Trypanosoma brucei*: timing of event markers and cytoskeletal modifications. *Phil. Trans. R. Soc. Lond. B.*, **323**, 573–588.
Sita,R. and Meldolesi,J. (1992) Endoplasmic reticulum: a dynamic patchwork of specialized subregions. *Mol. Biol. Cell*, **3**, 1067–1072.
Smith,T.K., Cottaz,S., Brimacombe,J.S. and Ferguson,M.A.J. (1996) Substrate specificity of the dolichol mannose-glucosaminyl-phosphatidylinositol α1-4 mannosyltransferase of the glycosyl-phosphatidylinositol biosynthetic pathway of African trypanosomes. *J. Biol. Chem.*, **271**, 6476–6482.
Smith,T.K., Milne,F.C., Sharma,D.K., Cossman,A., Brimacombe,J.S. and Ferguson,M.A.J. (1997) Early steps in glycosylphosphatidylinositol biosynthesis in *Leishmania major*. *Biochem. J.*, **325**, 393–400.
Staehelin,L.A. (1997) The plant ER: a dynamic organelle composed of a large number of discrete functional domains. *Plant J.*, **11**, 1151–1165.
Stieger,J. and Seebeck,T. (1986) Monoclonal antibodies against a 60 kDa phenothiazine-binding protein from *Trypanosoma brucei* can discriminate between different trypanosome species. *Mol. Biochem. Parasitol.*, **21**, 37–45.
Stevens,V.L. (1995) Biosynthesis of glycosylphosphatidylinositol membrane anchors. *Biochem. J.*, **310**, 361–370.
Takahashi,M., Inoue,N., Ohishi.K., Maeda,Y., Nakamura,N., Endo,Y., Fujita,T., Takeda,J. and Kinoshita,T. (1996) PIG-B, a membrane protein of the endoplasmic reticulum with a large lumenal domain is involved in transferring the third mannose residue of the GPI anchor. *EMBO J.*, **15**, 4254–4261.
Takeda,J. and Kinoshita,T. (1995) GPI-anchor biosynthesis. *Trends Biol. Sci.*, **20**, 367–371.
Turco,S.J. and Descoteaux,A. (1992) The lipophosphoglycan of *Leishmania* parasites. *Annu. Rev. Microbiol.*, **46**, 65–94.
van't Hof,W., Rodriguez,E. and Menon,A.K. (1995) Nonpolarized distribution of glycosylphosphatidylinositols in the plasma membrane of polarized Mardin–Darby canine kidney cells. *J. Biol. Chem.* **270**, 24150–24155.
Vertel,B.M., Velasco,A., LaFrance,S., Walters,L. and Kaczman-Daniel,K. (1989) Precursors of chondroitin sulfate proteoglycan are segregated within a subcompartment of the chondrocyte endoplasmic reticulum. *J. Cell Biol.*, **109**, 1827–1836.
Vickerman,K. (1969) On the surface coat and flagellar adhesion in Trypanosomes. *J. Cell Sci.*, **5**, 163–193.
Vidugiriene,J. and Menon,A.K. (1993) Early lipid intermediates in glycosyl-phosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. *J. Cell Biol.*, **121**, 987–996.
Vidugiriene,J. and Menon,A.K. (1994) The GPI anchor of cell-surface protein is synthesized on the cytoplasmic face of the endoplasmic reticulum. *J. Cell Biol.*, **127**, 333–341.
Watanabe,R., Kinoshita,T., Masaki,R., Yamamoto,A., Takeda,J. and Inoue,N. (1996) PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis form a protein complex in the endoplasmic reticulum. *J. Biol. Chem.*, **271**, 26868–26875.
Wessel,D. and Flugge,U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.*, **138**, 141–143.
Zawadzki,J.L., Scholz,C., Currie,G., Coombs,G.H. and McConville, M.J. (1998) The glycosylinositolphospholipids from *Leishmania* panamensis contain unusual glycan and lipid moieties. *J. Mol. Biol.*, **282**, 287–299.
Zilberstein,D. and Dwyer,D.M. (1984) Antidepressants cause lethal disruption of membrane function in the human protozoan parasite *Leishmania*. *Science*, **226**, 977–979.

Received March 3, 1999; revised and accepted May 14, 1999