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Use of the green fluorescent protein as a marker in transfected *Leishmania*

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Abstract

We have tested the suitability of the green fluorescent protein (GFP) of *Aequorea victoria* as a marker for studies of gene expression and protein targeting in the trypanosomatid parasite *Leishmania. Leishmania* promastigotes expressing GFP from episomal pXG vectors showed a bright green fluorescence distributed throughout the cell, readily distinguishable from control parasites. Transfection of a modified GFP gene containing GC-rich synonymous codons and the S65T mutation (GFP+) yielded a much higher fluorescence. FACS analysis revealed a clear quantitative separation between GFP-transfected and control parasites, with pXG-GFP+ transfectants showing fluorescence signals more than 100-fold background. Episomal DNAs could be recovered from small numbers of fixed cells, showing that GFP could be used as a convenient screenable marker for FACS separations. GFP was fused to the C-terminus of the LPG1 protein, which retained its ability to restore LPG expression when expressed in the *lpg*$^-$ R2D2 mutant of *L. donovani.* The LPG1(GFP) fusion was localized to a region situated between the nucleus and kinetoplast; its pattern was similar to that of LPG2, which is known to be located in the Golgi apparatus. This is notable as LPG1 participates in the biosynthesis of the glycan core of the LPG GPI anchor, whereas protein GPI anchor biosynthesis occurs in the endoplasmic reticulum. These studies suggest that the GFP will be a broadly useful marker in *Leishmania.*

Keywords: Fluorescent tag; Protozoan parasite; Glycolipid biosynthesis; secretory pathway; GPI anchors; Glycolipids; Lipophosphoglycan

Abbreviations: GFP, green fluorescent protein of *Aequorea victoria*; LPG, lipophosphoglycan; GPI, glycophosphatidylinositol; FACS, fluorescence-activated cell sorter.

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1. Introduction

The use of reporter molecules has become a standard method in molecular genetic analysis, including those now applicable to many proto-

zoan parasites. Proteins such as *E. coli* β-galactosidase are widely used to monitor gene expression in transient and stable transfection assays, or to trace the subcellular localization of engineered fusion proteins. Recently, the green fluorescent protein (GFP) of *Aequorea victoria* has been introduced as a convenient marker in eukaryotic organisms [1]. GFP can be readily used to monitor gene expression in several cellular compartments, and is active when expressed as N- or C-terminal fusions. Moreover, GFP is compatible with other fluorescent markers, and does not require other co-factors or cell-type or species-specific modifications for fluorescence [1]. Here we asked whether GFP could be successfully expressed in *Leishmania* promastigotes, and explored some potential applications in FACS analysis and protein localization.

2. Methods

2.1. Leishmania culture and transfection

Leishmania major was the wild-type LT252 clone CC-1 [2]. *L. donovani* was either wild-type 1S2D (line Ld4) or the *lpg*⁻ mutant R2D2 [3]. Clonal lines of transfected *Leishmania* were obtained by electroporation in the presence of appropriate DNA constructs and plating on semisolid M199 medium containing G418 and/or hygromycin B as described [2,4]. Prior to analysis, transfectant clones were usually selected with progressively increasing drug levels, to a final concentration of 1 mg/ml for G418 or 200 μg/ml for hygromycin B. The LPG phenotype of was tested by agglutination with the anti-LPG antibody CA7AE [5].

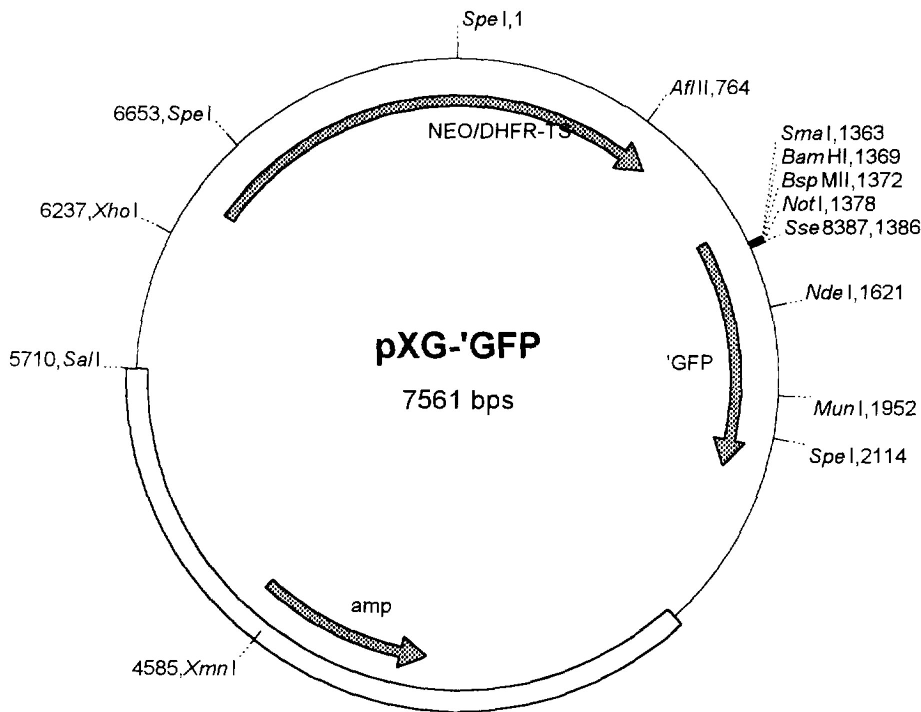
2.2. Molecular constructs

The pXG vector (lab strain B1288; the 'G' signifies 'general') is a derivative of pX63NEO [4]. It contains a 0.74-kb *Sal*I-*Sph*I fragment encompassing the splice acceptor and upstream poly(A) site for the 1.7-*l* R region mRNA [6], inserted downstream of the *Bam*HI expression site of pX63NEO (L. Borges and SMB, unpublished data). pXG shows a higher level of expression than previous pX vectors, especially in the amastigote stage. pXG-GFP (B2355) was made by insertion of the GFP-containing 0.7-kb *Kpn*I-*Eco*RI fragment from plasmid TU # 65 [1] into the *Sma*I site of pXG, after filling in with the Klenow fragment of DNA polymerase I. pXG-'GFP (strain B2295) contains a truncated version of the GFP, in which the normal start codon has been substituted with a polylinker (Fig. 1). For this construct, the oligos SMB 104 (5'cgggatccggagcggccgccctgcaggCTAGCAAAGGAGAA; lower case marks nucleotides not present in GFP) and SMB 105 (5'-cgagatCTATTTGTATAGTTCATC) were used in a PCR with the TU # 58 [1] template to generate a modified GFP cassette suitable for protein fusions. The product was digested with *Bam*HI and *Bgl*II, and inserted in the sense orientation into the *Bam*HI site of pXG. DNA sequence analysis revealed that the CTT leucine at codon 7 of GFP had been changed to CTC, which is fortuitously both synonymous and more common in *Leishmania* genes [7]. pXGH-LPG1(GFP) (strain B2305) contains (1) a 5.7-kb *Xmn*I fragment from pX63HYG-LPG1 containing the HYG resistance marker and the 5' end of LPG1 [8], (2) a 3.3-kb *Xmn*I-*Not*I fragment from pXG1-'GFP containing the bacterial vector and 'GFP sequences, and (3) a small PCR-derived fragment extending from the *LPG1 Xmn*I site through the LPG1 C-terminus, changing the sequence at the termination codon from CCC AGC TAA to CCC AGT gcg gcc gcc for fusion with the 'GFP cassette in pXG-'GFP (lower case bases represent an added *Not*I site). pXG-GFP+ (strain B2799) was made by blunt-end ligation of the GFP *Hin*dIII-*Not*I fragment of pSynGFP-S65T (E-C. Park and B. Seed, Massachusetts General Hospital) into the *Sma*I site of pXG.

2.3. Visualization of GFP in Leishmania

Live cells were viewed directly or after suspension in PBS (phosphate-buffered saline) containing 0.05% NaN_3. Ethanol fixation was performed by resuspending logarithmic phase cells at 5×10^6 parasites in 5 ml 1 × PSG (2 × PSG contains 1.3

A.



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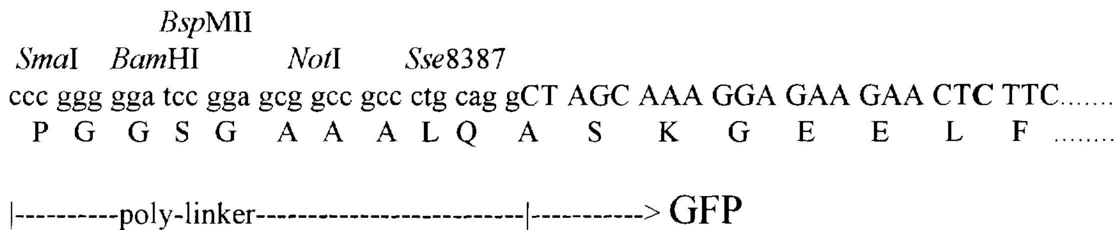

Fig. 1. (A) Restriction map for pXG-'GFP. The open region represents the bacterial vector (pSP6-T3). Filled arrows represent known or predicted mRNAs directed by the processing signals present in the *L. major* intergenic regions. *NEO/DHFR-TS* depicts the *NEO* resistance marker flanked by the *DHFR-TS* intergenic regions [2,6], while *'GFP* depicts the GFP protein fusion cassette, flanked on the 5' side by the *DHFR-TS* DST intergenic region and on the 3' side by the 1.7-*l* mRNA intergenic region [6]. 'Amp' marks the β-lactamase, which is only expressed in *E. coli*. All sites for the indicated restriction enzymes are shown. Recently we have designed an analogous construct with the GFP+ gene (H. Xu and SMB, unpublished). (B) Sequence of the polylinker of pXG-'GFP (the bold nucleotide marks a mutated leucine codon introduced into GFP as described in the Methods). A *trans*-splice acceptor site is located about 80 bp 5' of the sequence shown [6].

ml 5 M NaCl, 7.1 ml 1 M Na_2HPO_4, 0.4 ml 1 M NaH_2PO_4, 5 ml 20% glucose, and 36.2 ml H_20). One ml glycerol and 14 ml ethanol were added and the suspension was mixed, and incubated overnight at −20°C. Paraformaldehyde fixation was performed by washing cells twice in PBS, and resuspending at a density of 10^6/ml in 4% paraformaldehyde in PBS for 15–30 min. Cells were then washed twice in PBS. Formaldehyde fixation was performed by adding 0.6 ml of 37% formaldehyde to 5 ml of a logarithmic phase culture, and shaking for 1 h at 26°C. Cells were

washed twice in 5 ml of 1.1 M sorbitol, 0.1 M potassium phosphate, pH 6.5, and resuspended at 10^6/ml in the same solution.

For wet mount slides, VectaShield (Vector) was superior for GFP fluorescence, and 50% glycerol/PBS was superior for simultaneous GFP and Hoechst 33258 fluorescence. An Olympus BHS-BHT photomicrographic system was used, with either UG1 excitation and L-420 barrier filters (Hoechst 22538), or BP490 excitation and AF + O-515 barrier filters (GFP). To visualize the nuclear and kinetoplast DNA, cells were stained with Hoechst 33258 (1 μg/ml in PBS). Rabbit antibody to the Lm39 protein [9] was employed as a marker for the endoplasmic reticulum.

2.4. FACS analysis of Leishmania expressing GFP

L. major fixed by the methods above were analyzed with Becton Dickinson FACS Vantage or Calibur machines. Parasites were illuminated at 365, 457 or 488 nm, and fluorescence detected at 520 nm. While not optimal for either absorbance or emission of wild-type GFP [1], they are well suited for use with the S65T mutant contained in GFP+ and are available on most FACS machines.

2.5. Recovery of plasmids from transfected Leishmania

Varying numbers of *L. major* transfected with pXG-GFP were used. Total DNA was recovered [10] and resuspended in 100 μl 10 mM Tris, 1 mM EDTA, pH 7.4. Four μl of this preparation was combined with 0.02 ng of a chloramphenicol-resistant control plasmid (pBC-K+), and electroporated into *E. coli*; the transformed cells were divided equally and plated on either ampicilllin (100 μg/ml) or chloramphenicol (35 μg/ml) plates.

3. Results

3.1. Expression of GFP in Leishmania

A DNA cassette containing the coding region for the *Aequorea* GFP [1] was inserted into the *Leishmania* expression vector pXG (L. Borges and SMB, unpublished work), yielding pXG-GFP. After introduction into *L. major*, several clonal transfectants were examined for GFP expression under illumination with ultraviolet light. A clear green signal could be readily detected in motile living parasites, or after fixation by several methods (Fig. 2A). In a mixture of GFP-transfected and control *Leishmania*, the two types of cells could be readily distinguished (Fig. 2D–F). Within *Leishmania*, GFP was distributed throughout most of the cell, although it was possibly excluded from the mitochondrion (Fig. 2; compare panel A depicting GFP with panel B, showing staining with the DNA-specific dye Hoechst 33528).

3.2. FACS analysis of GFP expression

A powerful application of GFP would be in the analysis and recovery of parasites expressing GFP gene or protein fusions, perhaps expressed differentially under varying experimental conditions. To test the potential for this approach in *Leishmania*, we explored the use of several excitation wavelengths (365, 457 and 488 nm) with detection at 520 nm, and compared control and GFP-expressing *L. major*. These studies suggested that excitation at 488 nm and detection at 520 nm gave the maximum difference between the control and GFP-expressing transfectants. With all fixation methods tested, control parasites showed an average signal of 2–3 fluorescent units (FU), while the pXG-GFP transfected population grown in 1 mg/ml G418 showed an average of 20–40 FU (Fig. 2a–c). The pXG-GFP transfected *L. major* showed a broad distribution, which may reflect differences in plasmid copy number or the specific activity of GFP fluorescence, perhaps due to differences in auto-oxidation [11].

We tested a modified GFP protein cassette (termed GFP+ here) developed by E-C. Park and B. Seed (personal communication). GFP+ contains an S65T mutation [12] and was further

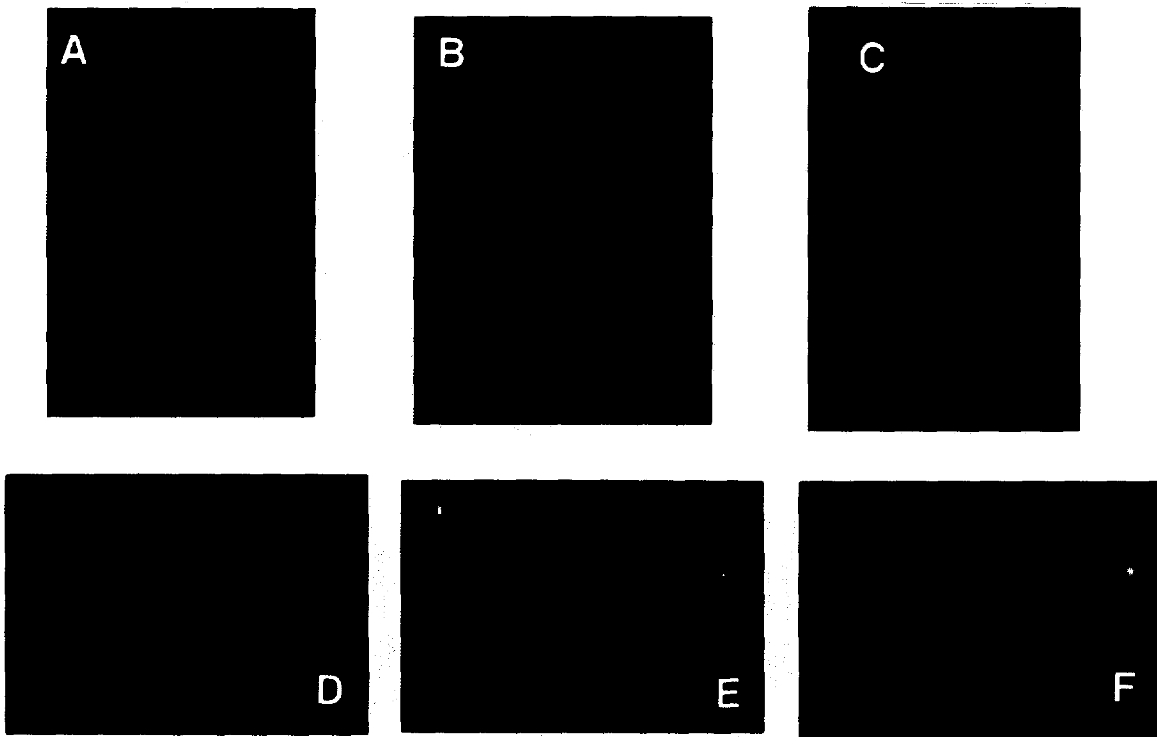

Fig. 2. Expression of GFP in transfected *Leishmania major*. (A,B) *L. major* transfected with pXG-GFP were fixed by paraformaldehyde treatment, and stained with Hoechst 33258. They were photographed under conditions specific for GFP fluorescence (A) or DNA (B) fluorescence. (C) *L. donovani* transfected with pXG-LPG1(GFP) were fixed by paraformaldehyde treatment, stained with Hoechst 33258, and photographed for both GFP (green) and DNA (blue). (D–F) *L. major* transfected with pXG-GFP were mixed 1:1 with wild-type *L. major*, fixed by paraformaldehyde treatment, and photographed for GFP fluorescence. Panel D was photographed for GFP only, panel E for DNA only, and panel F for both.

modified by substituting GC-rich synonymous codons. This seemed advisable since *Aequorea* GFP mostly contains AT rich synonomous codons [13], including several rarely used by *Leishmania* [7]. FACS analysis of transfectants grown at 20 μg/ml G418 showed that control cells exhibited a peak at 3 FU as before, while pXG-GFP transfectants grown at this lower G418 concentration exhibited a peak at 8 FU (Fig. 3D). Significantly, pXG-GFP+ transfectants showed a broad peak from 300 to 800 FU (Fig. 3D). Thus, the GFP+ cassette provides a greatly increased fluorescence signal, more than 100-fold background.

3.3. Recovery of plasmid DNA from small numbers of transfected Leishmania

The results above suggested that it should be possible to employ FACS methodology to identify GFP-expressing cells from a mixed population. In many situations, the next step would be to recover the molecular construct mediating GFP expression from the parasite. Previously, we showed that episomal cosmids can be readily recovered from live, transfected *Leishmania* [5,8,14,15]. These studies used large quantities of cells (10^8 or greater) beyond the recovery capacity of direct cell sorting. Moreover, the cell sorter facility cur-

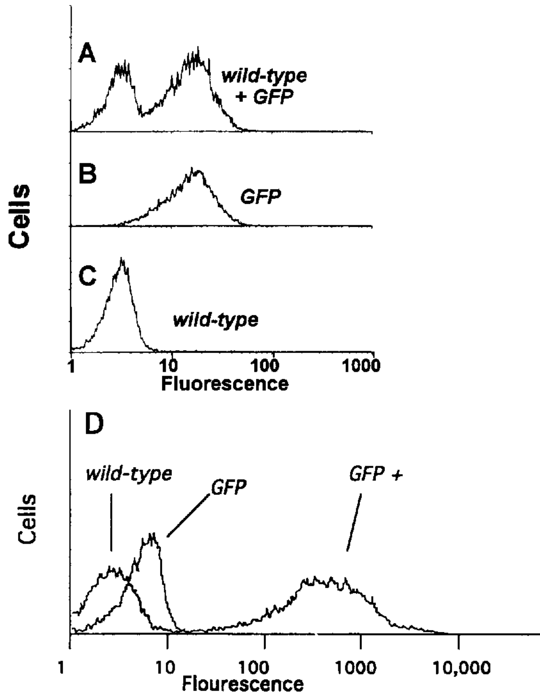

Fig. 3. FACS analysis of pXG-GFP transfected *L. major*. Cells were fixed with paraformaldehyde and analyzed by FACS, with illumination at 488 nm and detection at 520 nm. (A) A 50:50 mix of wild-type and pXG-GFP transfected *L. major* cultured in 1 mg/ml G418); (B) CC-1 transfected with pXG-GFP (cultured in 1 mg/ml G418); (C) wild-type CC-1 *L. major*. (D) FACS analysis of wild-type, pXG-GFP and pXG-GFP+ transfected *L. major* (transfectants grown in 20 μg/ml G418).

rently available to us did not permit analysis of live, virulent *Leishmania*, which precluded expanding a small GFP-positive population following sorting. We thus tested whether transfecting episomal DNAs could be recovered, prior to FACS separation, from small numbers of cells that had been killed and/or fixed. In these experiments, *L. major* promastigotes transfected with pXG-GFP were fixed with ethanol or killed with azide, and DNA recovered from varying numbers of cells using a simple mini-prep procedure. The recovery of plasmid DNA was monitored by transformation into *E. coli*. Plasmids could be readily recovered from as few as 10^4–10^5 fixed cells (Table 1), a number readily obtainable by direct FACS sorting. The yield from unfixed cells was somewhat higher (Table 1); when possible, live sorted cells could be cultured further to increase the yield. This study thus supports the feasibility of FACS methodology for the recovery of GFP transfected parasites from mixed populations following fixation and FACS separation.

3.4. Localization of an LPG1-GFP fusion protein

One of the major uses of the GFP is as a marker for cellular localization of fusion proteins [1]. To test this in *Leishmania* GFP was fused in-frame to the C-terminus of LPG1 [8], yielding the construct pXGH-LPG1(GFP). This or pXG-GFP was transfected into the R2D2 mutant of *L. donovani*, which contains a defect in LPG expression which can be rescued by expression of LPG1 [8]. Agglutination tests showed that R2D2 expressing LPG1(GFP), but not GFP alone, exhibited an LPG$^+$ phenotype by agglutination tests [5,8]. Thus, the C-terminal GFP tag did not compromise the functionality of LPG1.

GFP-expressing *L. donovani* showed uniform staining (not shown), identical to that obtained with *L. major* (Fig. 2A). In contrast, LPG1(GFP) transfectants showed localization of GFP to a small region situated between the nucleus and kinetoplast DNA (Fig. 2C). Notably, this area is

Table 1
Recovery of pXG-GFP plasmid from transfected *Leishmania major* fixed by different methods

Preparation method	Total ampicillin-resistant[a] colonies obtained from:			
	10^4 cells	10^5 cells	10^6 cells	10^7 cells
Ethanol	0	250	1500	24 000
Azide	50	450	2100	41 000
None (live)	nd	nd	nd	62 000

[a]Ampicillin resistance marks the recovery of the pXG-GFP plasmid. In these experiments, the transformation efficiency of the co-electroporated control plasmid was 8–11 × 10^7 chloramphenicol-resistant colonies/μg pBC-K−. n.d., not done.

known to be the site of the parasite Golgi apparatus, and the staining pattern was similar to that seen with LPG2 tagged with the influenza hemagglutinin epitope [15], another LPG biosynthetic protein implicated in the synthesis of the repeating phosphodisaccharide units of LPG. The LPG1(GFP) fluorescence pattern was clearly distinct from that obtained with a marker for the *Leishmania* endoplasmic reticulum, Lm39 protein ([9], data not shown).

4. Discussion

We have shown that the GFP protein tag exhibits many of the useful properties in *Leishmania* as in other eukaryotes. The signal is clear and distinctive, and intense enough to permit FACS analysis and localization of GFP-tagged proteins within the parasite. GFP-expressing DNAs may be readily recovered from live or fixed parasites under conditions mimicking the yields anticipated from direct FACS sorting.

From the emerging literature of GFP applications in other eukaryotes, a variety of uses in molecular and cellular studies of *Leishmania* may be contemplated. One feasible application would be to facilitate monitoring the fate or numbers of GFP-expressing *Leishmania* amastigotes during macrophage or animal infections. Another application involves the localization of cellular proteins, as exemplified here with LPG1. To this end, the *Leishmania* expression vector pXG-'GFP (and analogous constructs containing GFP+) provides a simple vehicle for rapidly engineering proteins bearing C-terminal GFP tags. Since some targeting signals occur at the C-terminus and may be affected by fusions at this site, it may be desirable to add an N-terminal GFP fusion vector to our repertoire. Lastly, FACS analysis would be suitable for enrichment of cells expressing the GFP marker from pools of transfected parasites, under conditions in which only some of the cells express the marker due to differences in expression or cellular localization.

Our data are consistent with a localization of LPG1 to the *Leishmania* Golgi apparatus. Confirmation of this result will require methods with more resolution, such as immuno-electron microscopy. Another essential control is the demonstration that the LPG1(GFP) fusion protein remains intact, as cleavage at the LPG1/GFP junction could lead to erroneous localization of the free GFP at a site distinct from LPG1.

Parasitic protozoans rely heavily upon GPI-anchored surface molecules in their interactions with the host [16]. *Leishmania* abundantly expresses a GPI-anchored protein, GP63, and a GPI-anchored glycan, the lipophosphoglycan (LPG), both of which play significant roles in the parasite infectious cycle [17]. For GPI-anchored proteins, it is believed that the GPI anchor is synthesized on the cytoplasmic face of the endoplasmic reticulum, and translocated to the lumen prior to protein addition [16]. Loss of GPI-anchored proteins in transfected *Leishmania* expressing the cytoplasmic trypanosome phospholipase C supports this view [18]. In contrast, these same transfectants show no reductions in LPG expression, suggesting that LPG GPI anchor biosynthesis occurs in another cellular compartment. Interestingly, comprehensive surveys of *Leishmania* GPI-anchored molecules suggest major divisions amongst the biochemical pathways leading to LPG, glycosyl-inositol-phospholipid (GIPL) and protein GPI anchor biosynthesis [19]. To these findings we may now add the observations that the LPG biosynthetic proteins LPG2 [15] and (tentatively) LPG1 occur within the parasite Golgi apparatus. From these data, it is reasonable to propose that the site of LPG biosynthesis differs from that of GPI-anchored proteins, and may lie within the parasite Golgi apparatus. Efforts to confirm this hypothesis are currently underway.

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