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Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*

Stephen M. Beverley and Salvatore J. Turco

Leishmania exploits several strategies to survive within the phagolysosome of vertebrate macrophages and be transmitted by sand fly vectors. Recent advances in functional genetic analysis provide a new avenue for identifying genes implicated in the infectious cycle of the parasite, such as those necessary for the synthesis and expression of the key surface glycoconjugate, lipophosphoglycan (LPG).

S.M. Beverley is in the Dept of Molecular Microbiology, Washington University Medical School, St Louis, MO 63110, USA; S.J. Turco is in the Dept of Biochemistry, University of Kentucky, Lexington, KY 40536, USA.*
**tel: +1 314 747 2630, fax: +1 314 747 2634, e-mail: beverley@borcim.wustl.edu*

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of human diseases, termed leishmaniasis. Depending on the species involved, leishmaniasis appears clinically in three forms: cutaneous, mucocutaneous and visceral, the last being fatal if untreated. The parasites have a remarkable capacity to avoid destruction in the hostile environments encountered in their life cycle, alternating between intracellular macrophage parasitism and extracellular life in the gut of the sand fly vector (Fig. 1). As with other microbial pathogens, the development of genetic tools for the study of these parasites promises to help unravel the molecular details of how they persevere under such harsh circumstances.

The state of *Leishmania* genetics

Since the first report of transient transfection in the related protozoan *Leptomonas*[1], great progress has occurred in the range of molecular genetic approaches available for the study of *Leishmania* and its trypanosome relatives. Table 1 summarizes current approaches and compares *Leishmania* with the reigning leader in eukaryotic genetics, the yeast *Saccharomyces cerevisiae*. The available methods include a variety of positive and negative markers, diverse episomal and integrating expression vectors, highly efficient homologous gene replacement, artificial chromosomes (LACs) and inducible expression systems. These techniques permit extensive testing of known parasite genes by reverse genetic methods.

An important limitation of reverse genetic approaches is that one must already have identified a gene, by any one of several routes (homology, expression, genome projects or immunoreactivity). As these properties do not guarantee that the target gene will turn out to be functionally 'interesting', the researcher is often placed in a position of expectancy about the phenotype of engineered mutations. In contrast, genetic approaches, which start with a mutant showing alterations in some important property, can provide some promise (again not absolute) that the genes involved will turn out to be 'interesting'. Moreover, genetic screens are not limited by the researcher's preconceptions as to which molecules are important; instead, the organism is given the opportunity to identify relevant processes without prejudice.

Leishmania and other trypanosomatids pose interesting challenges for prospective geneticists. First, sexual crosses are either impossible or difficult. Although the occasional occurrence of hybrid parasites has raised the prospect of experimental sexual exchange in *Leishmania*, population genetic approaches have not disclosed a high level of genetic exchange in nature[2]. Several attempts to generate crosses between marked parasites have proved unsuccessful (summarized in

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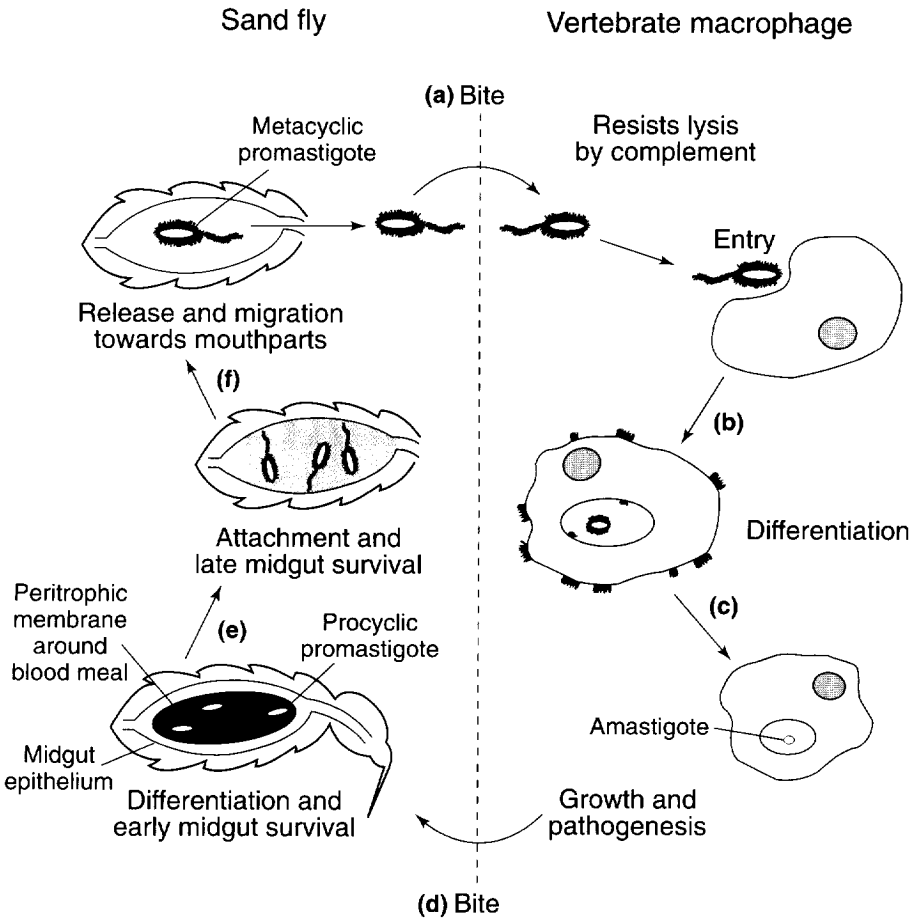

Fig. 1. The infectious cycle of *Leishmania* and the role of lipophosphoglycan (LPG). **(a)** Following an infectious bite, infective promastigotes (blue) must resist lysis by complement, a process mediated by LPG. **(b)** Parasites are then ingested by macrophages, which can recognize LPG and other parasite surface molecules, and are taken up into the phagolysosome. During this period, some of the LPG is deposited on the surface of the macrophage and internal vesicles, where it plays a role in modulating host signal transduction. **(c)** Soon after entry, the parasite differentiates into the amastigote form (yellow), which replicates and is responsible for the manifestations of disease. The parasitophorous vacuole is known to be acidic and to contain hydrolytic enzymes. Amastigotes do not synthesize LPG but do contain several glycoconjugates related to LPG (including proteophosphoglycan, acid phosphatase and glycophosphatidylinositol lipids). **(d)** Infected macrophages are taken up with the blood by a second sand fly bite and initially reside within the blood meal (shown in red) within the peritrophic membrane inside the sand fly midgut. Amastigotes released from the macrophage differentiate into the procyclic promastigote stage (green), where they rapidly acquire a coat of procyclic form LPG and begin to multiply. **(e)** Following rupture of the peritrophic membrane after about 2 days, procyclic promastigotes attach to the midgut wall through specific binding of LPG. **(f)** Once digestion of the blood meal is completed (about 5–7 days), the parasites cease dividing and differentiate into metacyclic promastigotes, which have a structurally altered LPG that is incapable of binding to the midgut wall. These highly virulent forms detach from the gut wall and migrate anteriorly in preparation for the next infectious bite.

Ref. 3), although one cross between lines bearing episomal selectable markers has recently been reported (A. Laban, pers. commun.). If this could be performed routinely with chromosomal markers, it would be tremendously useful. Second, *Leishmania* and trypanosomes are diploid organisms. This poses no problem for dominant gain-of-function mutations, which may require only a single activating mutation, but poses a considerable problem in generating loss-of-function mutations, as two alleles must be disrupted by independent mutations or by one mutation followed by some mechanism involving loss of heterozygosity[4]. Regardless of the mechanism, even after heavy mutagenesis *Leishmania* mutants are recovered at a low frequency, in the order of 10^{-7} (Refs 4–6). Experimentally, this means that selection experiments rather than screening assays must be used to identify mutants, placing a tremendous burden on those seeking to make avirulent mutants, whose phenotype is difficult to select for directly *in vivo* or *in vitro* in *Leishmania* using current methods (the number of mice required to screen for avirulent mutants would fill a large room!). It should be noted that other eukaryotic microorganisms are also 'asexual' diploids, including species of *Candida*[7], creating similar obstacles for researchers. Fortunately, there is a candidate virulence determinant in *Leishmania* whose properties made it an ideal starting point for genetic manipulations.

Lipophosphoglycan (LPG): a multi-functional virulence determinant

The promastigote form of *Leishmania* is covered with a thick glycocalyx, composed primarily of a single molecule termed lipophosphoglycan (LPG; see Fig. 2). LPG contains a repeating polymer of disaccharide–phosphate repeating units, anchored to the surface of the parasite by a glycophosphatidylinositol (GPI) anchor. In many ways, LPG seems to be functionally and structurally analogous to the lipopolysaccharide of many prokaryotes. The abundance of LPG on the parasite surface, the site of the primary interface with the host, suggests a central role for the glycoconjugate in its infectious cycle. So far, LPG has been implicated in binding and release of the parasite in the midgut of the sand fly, resistance to complement, binding and uptake by macrophages, modulating macrophage signal transduction, resistance to oxidative attack, and, ultimately, allowing the parasite to establish successful infections (Fig. 1). The properties of LPG and its role in the infectious cycle have been reviewed elsewhere[8,9].

Importantly, the structure of LPG and its dominance on the parasite surface lends itself to the development of powerful selective methods required to obtain mutants in a diploid organism. LPG is terminated with a heterogeneous group of capping sugars, one of which includes β-linked galactosyl residues that are capable

of binding to lectins such as ricin agglutinin. By subjecting heavily mutagenized *Leishmania donovani* to multiple rounds of agglutination with ricin and enriching for non-agglutinated cells, nonreactive parasites are readily obtained. These survivors lack detectable LPG (Ref. 6) and are thus termed *lpg*⁻ mutants. In *Leishmania major*, which possesses a more complex LPG with many side-chain galactose residues attached to the disaccharide–phosphate repeats (Fig. 2), lectin[10] or anti-LPG antibody[11] selections often yield parasites with an LPG containing structural or conformational alterations instead.

Table 1. Genetic tools in *Leishmania* and the yeast *Saccharomyces cerevisiae*

Property	*S. cerevisiae*	*Leishmania*
Colonies	Yes (1–2 days)	Yes (1–2 weeks)
Positive markers	Many	>6
Negative markers	Many	>4
Expression vectors	Episomal, chromosomal	Episomal, chromosomal[36–38]
Artificial chromosomes	Yes	Yes
Efficient gene targeting/ knockout	Yes	Yes[39–42]
Inducible expression	Yes	Emerging
Transposable elements	Yes	Yes[28]
Functional rescue	Yes	Yes[13,14,25,43]
Sex	Yes	'Chaste'
Ploidy	Diploid or haploid	Diploid

In *L. donovani*, *lpg*⁻ parasites could arise from the loss of any step of the LPG biosynthetic pathway (from addition of the galactosyl cap through to the membrane phosphatidylinositol anchor) (Fig. 2). The first four *lpg*⁻ mutants characterized (R2D2, C3PO, OB1 and JEDI) each show different defects in LPG structure, with R2D2 affecting the glycan core and the others affecting different aspects of the disaccharide–phosphate repeats (Table 2; Fig. 2). Many other *lpg*⁻ mutants have been obtained and are now being characterized. From the structure of LPG, we estimate that at least 25 distinct biosynthetic enzymes are necessary, and current data suggest that a similar number of proteins will be required for the correct compartmentalization and targeting of LPG to the cell surface. Thus, LPG genetics is still in its infancy.

With the availability of mutants, the next task was to develop a functional rescue methodology similar to that used by geneticists studying other microbial systems. A genomic library has been constructed in an extrachromosomal *Leishmania–Escherichia coli* shuttle vector, cLHYG (Ref. 12), which has a capacity of 40 kb and thus requires only 1000 cosmids to cover the 35–50-Mb *Leishmania* genome minimally. Following transfection into *lpg*⁻ mutants, parasites are identified by lectin or anti-LPG antibody selection by panning[12–14]. Analysis of the cosmids carried by these *lpg*⁺ transfectants has confirmed that they all encode genes affecting LPG biosynthesis.

Properties of LPG genes

Four *L. donovani* LPG genes have been characterized (Table 2; Fig. 2). These can be divided into two classes, based on their mode of action. LPG class I genes (*LPG1* and *LPG4A*) encode glycosyltransferases and other enzymes required for the biosynthesis of LPG linkages and/or components. These enzymes and the novel linkages they synthesize offer an exciting opportunity for glycobiologists studying enzyme specificity and action, as well as to pharmaceutical companies seeking to design neoglycoconjugates or control leishmaniasis. For example, *LPG1* appears to encode a glycosyltransferase required for the addition of galactosylfuranose (Gal_f) within the LPG core (Table 2; Fig. 2). Gal_f is common in many microbial pathogens (in various bacteria, including *Mycobacterium*, as well as in *Trypanosoma cruzi* and several pathogenic fungi) but is not found in mammals, thus making it a potential target for rational chemotherapy.

Class II LPG genes (*LPG2* and *LPG3*) encode proteins involved in the compartmentalization and assembly of LPG, guiding it through the secretory pathway to its final surface destination. *LPG2* encodes a GDP–mannose translocation activity that functions in the

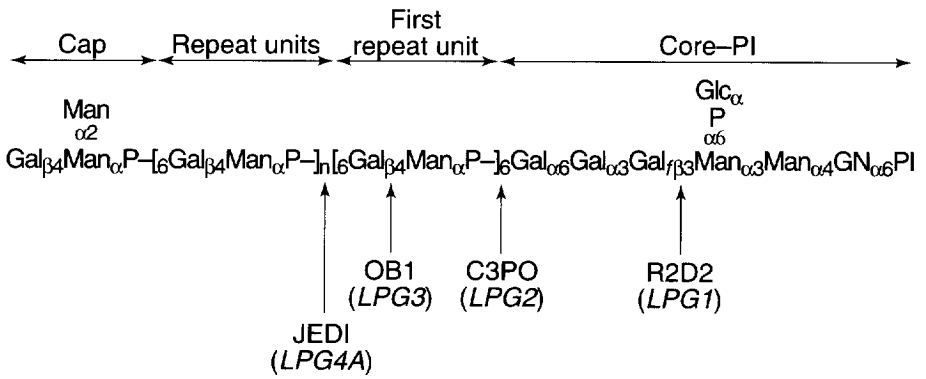

Fig. 2. Structure of lipophosphoglycan (LPG). The four domains of the *Leishmania donovani* LPG are (1) a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol anchor (PI), (2) a heptasaccharide glycan core, (3) multiple repeating disaccharide–phosphate units and (4) a small oligosaccharide cap. LPGs from all species of *Leishmania* have an identical lipid anchor and glycan core. The Gal–Man–PO_4 backbone of the repeating units is also conserved but additionally contains branching 'side-chain' sugars in other species of *Leishmania*. There can also be minor variations in cap structure. During metacyclogenesis (see Fig. 1), the number of repeating units on LPG approximately doubles and, in some species, additional modifications to the side-chain sugars occur. These play important roles in the ability of LPG to modulate binding and release from the midgut wall and in resistance to complement. The location of the defects in four *lpg*⁻ mutants (JEDI, OB1, R2D2 and C3PO) are shown. Abbreviations: Gal, galactose; Gal_f, galactosylfuranose; Glc, glucose; GN, glucosamine; Man, mannose; P, phosphate.

Table 2. LPG mutants and genes[a]

Mutant	Affected LPG domain	Gene	Mutation	Mode of rescue	Role	Refs
R2D2	LPG core	*LPG1*	Possible point mutations	Unknown	Putative Gal_f transferase	43,44
C3PO	Disaccharide–phosphate repeats	*LPG2*	Deletion	Complementation	Golgi GDP–mannose transport	13,15
OB1	Disaccharide–phosphate repeats	*LPG3*	Point mutations	Complementation	GRP94/HSP90 homolog; chaperone?	[b]
JEDI	Disaccharide–phosphate repeats	*LPG4A*	Deletion	Complementation	Putative mannosyl–phosphate transferase	[c]

[a]Abbreviations: LPG, lipophosphoglycan; Gal_f, galactosylfuranose.
[b]A. Descoteaux, S.J. Turco and S.M. Beverley, unpublished.
[c]H. Xu *et al.*, unpublished.

transport of this key substrate into the Golgi lumen, where GDP–mannose is used in assembling the disaccharide–phosphate repeats of LPG and other parasite molecules[13,15,16] (Fig. 2). *LPG3* encodes a member of the GRP94/HSP90 family of eukaryotic chaperones (A. Descoteaux, S.J. Turco and S.M. Beverley, unpublished; Table 2; Fig. 2). Unlike most chaperones, which usually show pleiotropic effects when mutated, *lpg3*$^-$ knockout mutants exhibit no defects other than loss of the characteristic disaccharide–phosphate repeats that are added to LPG and related molecules (Table 2; Figs 2,3). This suggests that the LPG3 chaperone has a restricted substrate specificity, which potentially recognizes nascent LPG motifs.

As LPG is developmentally regulated, being expressed at high levels in the promastigote but not the macrophage amastigote stage (Fig. 1), a third group (class III) of LPG mutations affecting regulatory pathways may exist. Theoretically, mutations that alter the promastigote developmental program towards that of the amastigote could confer an *lpg*$^-$ phenotype. Mutants of this class would be particularly informative, as little is known about the mechanism of stage-specific gene expression in *Leishmania*. Although no class III genes have yet been recovered, they may emerge when more *lpg*$^-$ mutations are characterized; a pessimistic alternative is that they may be lethal.

Examination of three of the LPG mutants has shown that C3PO and JEDI possess deletions of their respective *LPG* gene, whereas OB1 contains point mutations (R2D2 has not been studied intensively, although Southern blot analysis shows it to possess an unrearranged *LPG1* locus; Table 2). Interestingly, the two deletions in C3PO and JEDI appear to be homozygous, suggesting that mutation followed by loss of heterozygosity may be a common mechanism of diploid mutagenesis. In every case studied, *L. donovani lpg*$^-$ mutants appear to be loss-of-function mutants, and their rescue by cosmid transfection occurs by restoration of the defective gene by genetic complementation (rather than suppression).

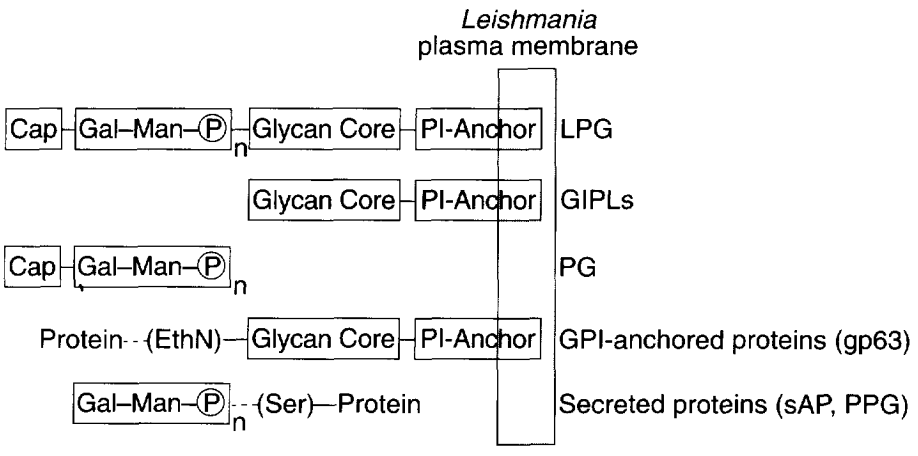

Fig. 3. Structure of *Leishmania* glycoconjugates related to lipophosphoglycan (LPG). *Leishmania* promastigotes display an abundant class of glycophosphatidylinositol lipids (GIPLs) and glycophosphatidylinositol (GPI)-anchored proteins, such as gp63, in addition to LPG, on their surface. Promastigotes secrete an acid phosphatase (sAP), a proteophosphoglycan (PPG) and a phosphoglycan (PG). All of these molecules contain domains related to LPG, although occasionally in modified form. The repeating disaccharide–phosphate units on the sAP, PPG and PG are identical to those contained on LPG. Whereas the phosphatidylinositol (PI) anchor of LPG is 1-*O*-alkyl-2-*lyso*-PI, it is 1-alkyl-2-acyl-PI in GPI-anchored proteins and some GIPLs. The glycan cores in LPG, GIPLs and GPI-anchored proteins vary in structure but all have the conserved Man(α4)GlcN(α6)*myo*-inositol motif that is characteristic of GPI-anchored molecules. Abbreviations: EthN, ethanolamine; Gal, galactose; Man, mannose; Ser, serine.

Is LPG a virulence determinant?

To confirm that a gene or molecule is a 'virulence' gene, a set of 'molecular' Koch's postulates should be satisfied[17,18]: (1) the molecule must be reasonably associated with pathogenicity or infectivity; (2) inactivation of the gene should lead to a significant loss of virulence and (3) restoration of gene function should fully restore pathogenicity. LPG readily satisfies the first criterion and, for the second, several *Leishmania lpg*$^-$ mutants are significantly compromised in their ability to survive in

macrophages, cause infections in animals or survive in the insect vector[10,19–22]. However, in none of these studies has the third postulate been applied, owing to a lack of cloned LPG biosynthetic genes. This poses a serious problem, as *Leishmania* (and many other pathogens) are known to lose virulence during *in vitro* culture[23]; moreover, *lpg*⁻ mutants are invariably obtained after heavy mutagenesis, the effects of which cannot be countered by sexual back-crossing. Culture or transfection-associated loss of virulence has also been a serious problem in the study of other presumptive virulence molecules in *Leishmania*, as the 'add-back' of the gene to the mutant frequently fails to restore virulence fully[24]. The solution to this problem is an important area for future studies.

Preliminary studies of some *lpg*⁻ mutants suggest that they do not satisfy the third postulate above. These mutants were obtained following mutagenesis and selection, or by targeted gene knockouts, in either *L. major* or *L. donovani*. Thus, for these mutants/genes a role (or lack thereof) in the infectious cycle cannot be proven. However, other *lpg*⁻ mutations do pass the third postulate, as their behavior in animal infectivity or fly survival tests is restored nearly or entirely to wild-type levels following restoration of *LPG* gene function. These findings emphasize the need for a rigorous examination of 'cause-and-effect' when studying asexual diploid parasites. The development of inducible expression systems will simplify this task considerably.

One complication in the study of *Leishmania* glycoconjugates is that LPG components appear on molecules other than LPG. For example, the disaccharide–phosphate repeats occur on secreted proteins, such as acid phosphatase (sAP) and proteophosphoglycan (PPG), and as a secretory polymer (PG; Fig. 3). Examination of *lpg2*⁻ mutants (C3PO or homozygous knockouts) reveals that they have lost all disaccharide–phosphate repeat-bearing molecules[13]. Thus, the phenotype of these mutants should be more precisely attributed to loss of repeats, rather than LPG alone. Similar concerns apply to other LPG components, such as the glycan core or phosphatidylinositol anchor (Fig. 2). As more LPG genes are identified, it seems likely that ones specifically affecting LPG domains will emerge, permitting more discrimination between the roles of these glycoconjugates.

New horizons for functional gene identification

So far, it has always been possible to perform successful functional genetic rescue if loss-of-function mutations can be obtained. Mutant hunts can be performed directly on wild-type parasites or on parasite populations bearing engineered reporter molecules designed to reveal prospective mutant phenotypes. In this way, a mutant (*gim1-1*) and then the gene (*GIM1*) affecting uptake of glycosomal proteins has been obtained[25]. This study illustrates the potential range for functional approaches through mass screening; although no selective method existed for the wild-type phenotype, examination of several thousand individual cosmid-transfected colonies has permitted identification of the one bearing *GIM1*.

Questions for future research

- What approaches could be used to increase the frequency of mutants in asexual diploids such as *Leishmania*?
- What are the forces and affected genes involved in loss of virulence during prolonged culture?
- How is lipophosphoglycan (LPG) synthesis compartmentalized within the cell, relative to enzymes involved in protein glycophosphatidylinositol anchor biosynthesis?
- What regulates LPG synthesis during development?
- Could similar approaches be used to identify mutants/genes affecting the synthesis of glycoconjugates found on the surfaces of other parasites, such as *Trypanosoma cruzi*, *Entamoeba* and *Trichomonas*?

The need for defined mutations and the difficulties in their recovery in the asexual diploid parasite *Leishmania* are strategic limitations at present. Thus, the development of methods to facilitate the identification of relevant mutants is a critical need. Basic studies of mutation, recombination, loss of heterozygosity, chromosome stability and ploidy, and treatments that could modulate these in a useful manner, will provide important insights into overcoming these obstacles.

There are other routes to the functional identification of 'interesting' genes currently under development in studies on *Leishmania*. One of these is multi-copy suppression, where overexpression or inappropriate expression of a wild-type protein bypasses a mutational defect. As episomal vectors such as cLHYG are present in multiple copies[12], transfected cosmid libraries are ideally suited to this mode of rescue. An obvious application of this strategy is in the identification of drug resistance genes, whose detection following gene amplification has been a popular topic in *Leishmania* genetics for some time[26,27]. Indeed, we have isolated numerous genes implicated in resistance to a wide spectrum of selective agents using this approach (P.C. Cotrim and S.M. Beverley, unpublished). Similarly, identification of gain-of-function mutations can be effected by transfection of cosmid libraries derived from mutant line DNA into wild-type lines, followed by appropriate selections.

Another approach is to use gene fusions. Recently, an active *Drosophila* transposable element, *mariner*, has been introduced into *Leishmania* in an active form[28]. This 'designer' insertional mutagen offers the possibility of generating 'tagged' gain-of-function or loss-of-function mutations, as well as gene fusions to drug resistance genes and reporter molecules, such as the green fluorescent protein (GFP; Ref. 29). GFP fusions in particular can be selected in various ways to yield molecules that show interesting patterns of stage-specific expression and/or cellular localization[30,31].

Conclusions

Genetic methods in *Leishmania* have progressed to the point that a variety of screens and selections designed to yield genes likely to play important roles in the infectious cycle can now be contemplated. Similar efforts are under way for other protozoan parasites, including the related kinetoplastid protozoans *Trypanosoma brucei* and *T. cruzi*, the apicomplexan parasites

Plasmodium and *Toxoplasma*, *Entamoeba*, *Giardia* and *Trichomonas*. In some of these parasites, genetic methods are well advanced and the feasibility of functional rescue has been clearly illustrated[32–35]. Although each of these parasites has its own peculiarities and hurdles, it seems likely that in the near future our understanding of virulence in protozoan parasites is likely to undergo a revolution.

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