Cloning and structure–function analysis of the *Leishmania donovani* kinetoplastid membrane protein-11

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This report describes the complete translated gene sequence, predicted secondary structure and lipid bilayer association of a novel kinetoplastid membrane protein (KMP-11) from *Leishmania donovani* promastigotes. KMP-11 was previously referred to as the lipophosphoglycan-associated protein (LPGAP). The isolation, species distribution and chemical characterization, including a partial protein sequence analysis and post-translational modifications, of this major membrane component have been described [Jardim, Funk, Caprioli and Olafson (1995) Biochem. J. **305**, 307–313]. C.d. measurements of KMP-11 indicated a very high helical content estimated to be approximately 86% in trifluoroethanol. This was in agreement with computer-based secondary-structure analyses which predicted KMP-11 to be almost exclusively α-helical, with the protein adopting a helix–loop–helix motif. Arrangement of the residues located in the putative helical regions on an Edmundson helical wheel showed that this molecule could have a strongly amphipathic conformation and provided an explanation for how such a highly charged protein might be inserted into the plasma membrane. Evidence in support of KMP-11 association with lipid bilayers was provided by showing that KMP-11 could mediate carboxyfluorescein release from liposomes. These findings suggested that KMP-11 may function in part to increase bilayer pressure, stabilizing molecules such as lipophosphoglycan within the parasite pellicular membrane.

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. Depending on the species, the disease symptomatology may range from a self-healing skin lesion to the fatal visceral form commonly known as kala-azar. Recent attempts to develop molecularly defined vaccines for these maladies have focused on major parasite cell surface molecules, including the metalloproteinase gp63 [1–3] and a lipophosphoglycan (LPG). The latter glycoconjugate has been implicated as a key molecule in the internalization and subsequent survival of parasites within the macrophage [4] and its abundance on the promastigote surface has made it an attractive vaccine candidate [5]. Whereas protective immunity was reported with LPG in BALB/c mice [6] and circulating lymphocytes from human leishmaniasis patients were shown to proliferate on exposure to LPG [7,8], the mechanism by which a glycan could affect such a response was difficult to rationalize in view of the present structural understanding of major histocompatibility complex–T-cell epitope interactions. Although several reports have indicated that LPG could protect against cutaneous disease and that this immunity could be adoptively transferred with $L3T4^+$ splenic T-cells [1,6], the possibility that this could be attributed to contaminating proteins in LPG preparations was not rigorously tested. Indeed, Mendonça et al. [8] have shown that treatment of LPG with protease K eliminated all human circulating lymphocyte proliferation in cells from American cutaneous leishmaniasis patients. At the same time an 11 kDa protein that co-purified with LPG was isolated in this laboratory and designated a lipophosphoglycan-associated protein (LPGAP). This protein, referred to here as the kinetoplastid membrane protein-11 (KMP-11), has been shown to be a strong stimulator of T-cell proliferation which cross-reacts with monoclonal antibodies raised against LPG preparations [2]. Results described in the preceding paper [9] show that KMP-11 is a major molecule on the surface of all *Leishmania* parasites considered to date and a broad species distribution has been found in kinetoplastid protozoa (C. Stebeck, R. P. Beecroft, B. N. Singh, A. Jardim, R. W. Olafson, K. D. Prenevost and T. W. Pearson, unpublished work). The present study is a continuation of the former work, providing details of the cloning, DNA sequencing and gene structure of KMP-11, together with a computer-predicted secondary structure and evidence for a hypothetical role in modulating the stability of LPG in the parasite membrane.

MATERIALS AND METHODS

Parasites

Leishmania donovani LD3 promastigotes, obtained from Dr. S. Turco (University of Kentucky, Lexington, KT, U.S.A.), were derived from the 1S2D clone by hamster passage. Promastigotes were cultured as described in preceding paper [9].

Screening of an *L. donovani* promastigote genomic DNA cosmid library for KMP-11

Genomic DNA was isolated from promastigotes by the method of Blin and Stafford [10] and the library was constructed using the Stratagene Supercos 1 cosmid vector as outlined in the supplier's protocol (Stratagene, La Jolla, CA, U.S.A.). A 45 bp oligonucleotide probe (ATGATCAAGGAGCACAC$^{G}/_{C}$GAGAAGTTCAACAAGAAGATGCACGAG) was synthesized (Calgary Oligonucleotide Service, Calgary, Alta., Canada) to amino acid residues 54–68 of KMP-11, using the preferred codon

Abbreviations used: LPG, lipophosphoglycan; KMP-11, kinetoplastid membrane protein-11.
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bias of *Leishmania* genes [11]. The probe was labelled with T4 polynucleotide kinase [12] [Pharmacia (Canada) Inc., Baie d'Urfe, Que., Canada] and [γ-^{32}P]ATP (Dupont Canada Inc., Mississauga, Ont., Canada). Approx. 2000 cosmid clones were plated on a nylon filter (150 mm; Schleicher & Schuell, Keene, NH, U.S.A.) and duplicate filters prepared. Filters were hybridized in a solution made up of 20 × SSC (1 × SSC = 0.15 M NaCl + 0.015 M sodium citrate), 5 ml of water, 7.5 ml of formamide, 2.5 ml of 100 × Denhardt's (Denhardt's = 2 g of Ficoll type 400, 2 g of polyvinylpyrrolidone and 2 g of BSA in 100 ml of H_2O), 0.5 ml of 10% SDS, 1 ml of 0.5 M sodium phosphate, pH 6.5, 0.5 ml of 10 mg/ml salmon sperm DNA and probe (1.2×10^6 c.p.m./ml; specific radioactivity 4×10^7 c.p.m./μg) and hybridized overnight at 42 °C with constant shaking. Filters were washed three times for 20 min with 100 ml of 2 × SSC/0.1% SDS at 42 °C, air-dried and autoradiographed.

Southern-blot analysis of genomic DNA for KMP-11 gene

Promastigote DNA (40 μg) was digested with 50 units of *Bcl*I, *Eco*RI, *Nco*I, *Sac*I, *Sal*I or *Xho*I overnight at 37 °C. Digests were separated on a 0.8% agarose gel, blotted to nitrocellulose membrane by capillary action and probed with the KMP-11 45 bp oligonucleotide probe as described above.

C.d. measurements

C.d. spectra were recorded on a Jasco J-700 spectropolarimeter (Jasco Inc., Easton, MD, U.S.A.) calibrated with ammonium (1*S*)-(+)-10-camphorsulphonate [13] using a cell path length of 0.02 cm. Octyl-Sepharose-purified KMP-11 [2] was dissolved in either 20 mM Tris/HCl, pH 7.8, or 10 mM Tris/HCl, pH 7.8/50% trifluoroethanol at a concentration of 320 μg/ml. The c.d. spectra of KMP-11 were analysed for secondary-structure elements using the Contin program version 1.0 [14].

Preparation of carboxyfluorescein-loaded small unilamellar vesicles

Unilamellar liposomes were loaded with 240 mM carboxyfluorescein in PBS by the method of Weinstein et al. [15] using the phospholipid distearoylphosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.). Free carboxyfluorescein was removed by gel-permeation chromatography on a Sephadex G-75 column (2 cm × 25 cm) equilibrated with 100 mM Tris/HCl, pH 7.5, containing 150 mM NaCl. To verify that the disruption of the liposomes was not due to extraneous glycolipids present in KMP-11 preparations, control assays were conducted by digesting KMP-11 with protease K at a 1:1 substrate/protease ratio. Fluorescence dequenching was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm.

RESULTS

Isolation and characterization of the *lpgap* gene

In the preceding paper [9], *kmp-11* was reported to have a blocked N-terminus precluding complete primary-structure elucidation by Edman degradation. Nonetheless, the nearly complete protein sequence was readily used to synthesize an oligonucleotide and probe a genomic library. Thus a 3.4 kb *Sac*I fragment from a cosmid clone was subcloned into a pBluescript plasmid, and limited nucleotide sequencing of the protein-coding portion revealed a single open reading frame of 276 bp with a molecular mass of 11 kDa. The translated product matched perfectly with the corresponding portion of the protein sequenced earlier by Edman degradation and the calculated pI of 4.9 agreed

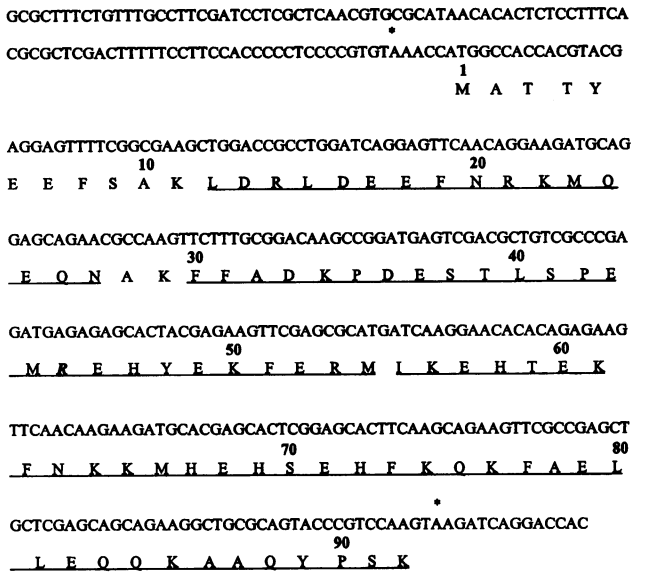

Figure 1 Protein and DNA sequence of KMP-11 from *L. donovani* promastigotes

Regions sequenced by Edman degradation chemistry are underlined. The gene sequence was determined by limited sequencing of a 3.4 kb *Sac*I fragment subcloned into the pBluescript vector. Asterisks indicate termination codons.

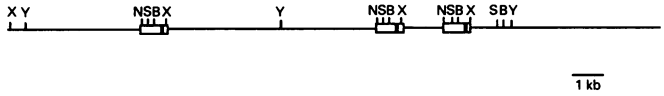

Figure 2 Restriction map of the *kmp-11* gene locus generated from genomic DNA and cosmid clone Southern-blot analysis

Shaded area indicates hybridization site for the KMP-11 45 bp oligonucleotide probe. B, *Bcl*I; E, *Eco*RI; N, *Nco*I; Y, *Sac*I; S, *Sal*I; X, *Xho*I.

well with that determined by isoelectric focusing (4.8) [9] confirming the acidic nature of this protein predicted by amino acid analysis. The complete gene sequence (*kmp-11*) is shown with the aligned protein sequence in Figure 1. It was significant that an in-frame termination codon was present 3 bp 5′ to the ATG initiation codon, indicating that the small size of KMP-11 cannot be ascribed to the proteolytic degradation of a larger polypeptide as originally proposed [2].

Experiments were then conducted to elucidate the gene arrangement for *L. donovani kmp-11*, as restriction maps had suggested several copies were present but were unable to distinguish between tandemly repeated genes and multiple copies with variable-sized intergenic regions. Southern-blot analysis of both genomic DNA and cosmid clones, digested with restriction endonucleases having only single cleavage sites within the *kmp-11* gene, showed that the *kmp-11* locus contained three gene copies. These were separated by the intergenic region of 1.9 and 3.5 kb indicating that *kmp-11* genes were not tandemly repeated (Figure 2).

Prediction of KMP-11 secondary structure

Determination of KMP-11 secondary structure was initially undertaken by analysing the protein primary structure with the empirical relations of both Garnier et al. [16] and Chou and Fasman [17]. Both methods predicted an unusually high α-helical

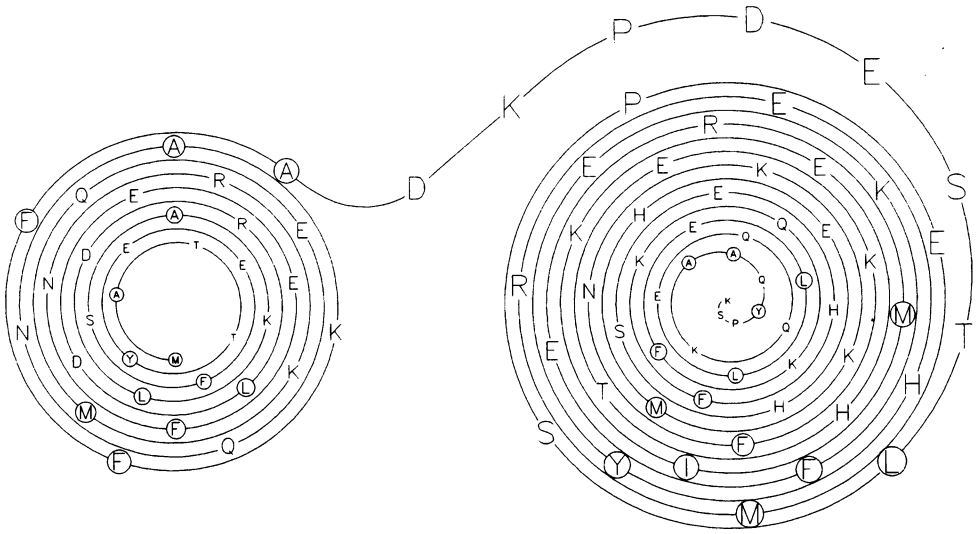

Figure 3 Predicted secondary structure of KMP-11

A schematic representation of KMP-11 secondary structure illustrating the proposed amphipathic nature of the two helices is shown. Hydrophobic residues are circled.

Table 1 Secondary structure of *L. donovani* promastigote KMP-11 deduced from c.d. measurements

	Secondary-structure elements (%)			
Solvent system	α-Helix	β-Sheet	β-Turn	Remainder
20 mM Tris/HCl, pH 7.8	53	9	2	36
50% Trifluoroethanol	86	12	0	2

content (91%) with two long helices containing 33 and 41 residues respectively. A random-coil segment was predicted to lie between the two helical regions as well as at the C-terminus, producing a helix–turn–helix motif (Figure 3) [16]. When the helical segments of this hairpin-like structure were plotted on an α-helical wheel, both were shown to have amphipathic character, with approximately one-third of the surface of each helix being hydrophobic and the remainder highly charged. A structure consistent with these findings is shown in Figure 3.

C.d. measurements of the *L. donovani* promastigote KMP-11 indicated that the purified protein adopted primarily α-helix (53%) and random-coil (36%) conformations in aqueous buffers (Table 1). However, the c.d. spectra of KMP-11 recorded in 50% trifluoroethanol, an α-helix-potentiating solvent, yielded results in close agreement with the structures predicted by both the Garnier and Chou–Fasman algorithms. In this organic solvent the α-helical content of KMP-11 was dramatically increased to 86%.

Homology search against the protein sequence database

Sequence-alignment analysis using the FASTA algorithm [18] and the available protein database identified apolipoprotein A-I and apolipoprotein A-IV as the optimal scoring proteins (Figure 4). The statistical significance of these homologies was assessed by the Pearson and Lipman RDF program [18]. Apolipoproteins A-I and A-IV gave optimized RDF values of 9.6 and 11.7 standard deviations above the mean of 20 randomly permutated sequences respectively which is diagnostic of the two molecules being significantly homologous with KMP-11.

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KMP-11                          L S P E M R E H Y E K F E R M I K E H T E K F N K K M H E H S E H F K Q K
                                L     +       E + F   R   +       E   F N K   +       E   + + Q K
Human apolipoprotein A-IV       L G G H L D Q Q V E E F R R R V E P Y G E N F N K A L V Q Q M E Q L R Q K

KMP-11                          D E S T L S P E M R E H Y E K F E R M I K E H T E K F N K K M H E H S E H F K Q K F A E L L E Q
                                +   + L   E M +   + E +     +       + F + K K + H E   E + + Q K + A L E
Bovine apolipoprotein A-I       E T A S L R Q E M H K D L E E V K Q K V Q P Y L D E F Q K K W H E E V E I Y R Q K V A P L G E E
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Figure 4 Sequence alignment of shared homologous region between KMP-11 and bovine apolipoprotein A-I and human apolipoprotein A-IV

Plus signs indicate conservative homologous replacements involving only single base substitutions.

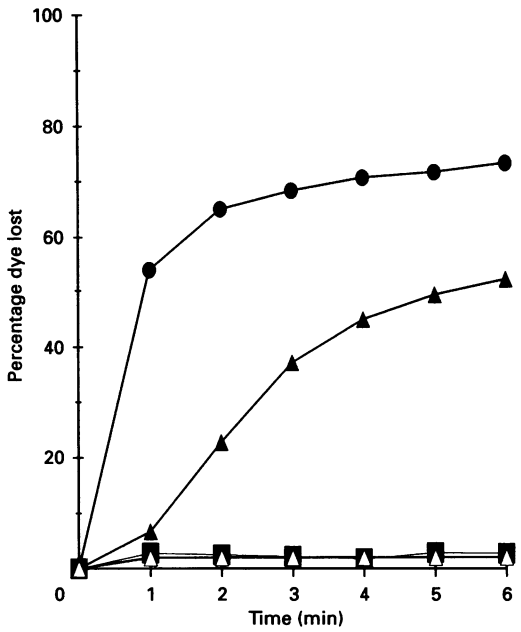

Figure 5 Effects of KMP-11 on carboxyfluorescein-loaded distearoylphosphatidylcholine vesicles

The rate of dye efflux was monitored by fluorescence dequenching: ●, untreated KMP-11; ▲, KMP-11 digested for 1 h with protease K; △, KMP-11 digested for 20 h with protease K; ▨, protease K alone.

KMP-11 association with small unilamellar vesicles

The predicted amphipathic nature of KMP-11, together with the apolipoprotein A-I and A-IV sequence homologies, suggested that the molecule may exhibit functionally important surface-active properties. The carboxyfluorescein technique [15], previously used in studies of apolipoprotein A-I synthetic peptides [19,20], was employed to evaluate the potential of KMP-11 insertion into a lipid bilayer. Distearoylphosphatidylcholine vesicles loaded with carboxyfluorescein were extremely stable, with less than 1% of the entrapped dye escaping after a 1 h incubation at room temperature. Lysis of the vesicles with 1% SDS was equivalent to 100% fluorescence. When KMP-11 was added to the liposome suspension to a final concentration of 20 μg/ml, a rapid release of carboxyfluorescein was noted with a $t_{1/2}$ of 12–15 s (Figure 5). To verify that the perturbation of the vesicle membranes was induced by protein and not a contaminating glycolipid, KMP-11 was digested with proteinase K. Pretreatment of KMP-11 for 1 h with protease K increased the observed $t_{1/2}$ to 120–130 s and was accompanied by a decrease in total fluorescence from 75 to 50% over a period of 5 min. Digestion of KMP-11 for 20 h eliminated all dequenching activity. This observation confirmed that KMP-11 and not lipid induced disruption of the liposome bilayer with subsequent release of carboxyfluorescein, supporting the predicted amphipathic structure for KMP-11 and highlighting a unique property of this molecule.

DISCUSSION

The *L. donovani* KMP-11 which is co-isolated with the glycoconjugate LPG has been shown to be the antigenic component responsible for the stimulation of both murine T-cells [2,21] and peripheral blood lymphocytes from patients recovered from New World mucocutaneous or visceral leishmaniasis [8,22]. On SDS/PAGE the purified protein migrates as multiple bands ranging in molecular mass from 11 to 40 kDa all sharing common determinants recognized by monoclonal antibodies L98 and L157 [2]. The latter observation was initially ascribed to proteolytic degradation of a larger parent molecule to yield the dominant 11 kDa protein species. However, results presented here clearly indicate that *kmp-11* genes only encode an 11 kDa protein. Evidently, the polydisperse nature observed on electrophoresis is due to an unusual electrophoretic behaviour attributed to either acid-labile post-translational modifications or aggregation of the KMP-11 molecule. The oversight of such an abundant membrane molecule by previous workers can therefore be attributed to this unusual behaviour in the presence of detergent. No doubt the asymmetric charge density in this amphipathic molecule plays a role here as the polydisperse behaviour is eliminated at acidic pH, allowing for a single band on SDS/polyacrylamide gels.

Two notable features of the translated protein were the absence of valine, glycine, tryptophan and cysteine residues together with a high charge density weighted towards acidic residues. As a secondary-structure prediction based on both the Garnier [16] and the Chou–Fasman [17] algorithms clearly predicted a helix–coil–helix motif, we were encouraged by the unusually low pI to consider the relative positions of polar and apolar residues in the predicted helical sequences. This analysis indicated an amphipathic compound and suggested that KMP-11 had the potential of forming a hairpin-like structure with both hydrophilic and hydrophobic surfaces. The latter occupied approximately one-third of the putative helical structure and would be expected to facilitate a strong interaction with lipid bilayers.

Evidence in favour of the above hypothetical folding model was provided by c.d. analysis of KMP-11, which indicated a high propensity for α-helical structure in an aqueous environment. This was significantly enhanced by addition of the organic solvent trifluoroethanol [23]. It is possible that the latter solvent more closely approximates to the apolar phospholipid bilayer environment expected for KMP-11 membrane association, resulting in the observed increase in helical structure [24].

As already indicated in the preceding paper [9], a minor proteolytic fragment (IVP) was consistently present in different KMP-11 preparations and corresponded to the entire KMP-11 C-terminus, including residues 32–92. The specificity for this cleavage site, together with the cell surface location of KMP-11, made the glycosylphosphatidylinositol-anchored metalloprotease gp63 [25,26] a likely candidate protease for this cleavage. Furthermore, on application of the secondary-structure algorithms to the complete protein sequence, it was predicted that the segment consistently cleaved would be situated in a random-coil configuration making it relatively susceptible to proteolytic attack. Whether this is a processing event of significance to the parasite, as discussed later, or merely an interesting coincidence, is a subject of investigation.

An indication of the potential biological role of KMP-11 may be inferred from homologies detected by protein-sequence-alignment analysis with the available protein database. Bovine apolipoprotein A-I and apolipoprotein A-IV were shown to be homologous with KMP-11. Apolipoprotein A-I is the major component of the high-density lipoprotein cholesterol-transport particle and has been suggested to enhance packing by filling spaces on the particle monolayer created by the phospholipid polar head groups and cholesterol [27]. Unlike apolipoprotein A-I, apolipoprotein A-IV has been less extensively studied. However, a structural feature shared by these two lipoproteins is a 22-amino acid segment repeated six times with a strong potential for amphipathic α-helix formation [28] similar to that predicted for

KMP-11. Work by Yokoyama et al. [29] has shown that synthetic peptides corresponding to one or two tandemly repeating apolipoprotein sequences not only favour helical conformations but also closely approximate the surface active properties observed for native apolipoprotein A-I on egg yolk phosphatidylcholine monolayers or unilamellar vesicles. The regions of homology KMP-11 and the apolipoproteins align with these amphipathic domains. It is notable that, except for helix length, the predicted conformation adopted by KMP-11 mirrors the synthetic peptide model derived for the apolipoprotein–lipid interaction [29]. This folding pattern for KMP-11 is consistent with the experimental data which show KMP-11 to be an extremely polar membrane-associated ^{125}I-surface-labelling protein.

The ability of KMP-11 to bind lipid was initially suggested by the tight association with LPG *in vitro*. Subsequent studies have helped to corroborate this supposition. For example, in an aqueous environment LPG would favour aggregation into mixed micelles containing KMP-11. Preliminary investigations have shown that the LPG–KMP-11 complex is eluted from gel-permeation chromatography columns with a molecular mass in excess of 150 kDa and that treatment of this complex with either phospholipase C or the detergent CHAPS allows both molecules to be resolved independently (A. Jardim, S. Hanson, B. Ullman, W. D. McCubbin, C. M. Kay and R. W. Olafson, unpublished work). These findings are consistent with an apolar stabilization of the complex. Further evidence demonstrating the affinity of KMP-11 for lipids was obtained from a KMP-11-mediated release of carboxyfluorescein from carboxyfluorescein-loaded liposomes. Although as yet the precise mechanism by which surface-active proteins are inserted into the bilayer is not fully understood, it is clear from this study that the protein perturbs the lipid bilayer sufficiently to allow carboxyfluorescein to escape [15].

Should KMP-11 be associated with LPG *in vivo*, the above marked affinity for lipid bilayers may have a bearing on the manner in which this association is made. The number of LPG molecules on the promastigote surface has been estimated to be 1×10^6–5×10^6/cell [30,31] forming a protective anionic barrier shielding the parasite membrane from harsh enzymic environments. It has been previously demonstrated that the state of the lipid bilayer is sensitive to the charge of ionizable phospholipid or glycolipid head groups causing destabilization of the membrane which is reflected in a decrease in the transition-phase temperature [32,33]. In this connection, it is plausible that the repulsive forces presented by the highly negatively charged LPG molecule would induce a lateral expansion and destabilization of the membrane causing an increase in the fluidity of the phospholipid bilayer [34]. Although some of these distortions may be mitigated by the long C_{24} to C_{26} alkyl chains of LPG [6,30], this may well be offset by the further instability inherent in the lysophospholipid structure. It seems possible therefore that the overall lipid bilayer pressure may be more precisely regulated by KMP-11 in a role analogous to that of apolipoprotein A-I. Although this is discussed here in the context of a putative membrane association with LPG, this function may well be more general in effect.

A striking feature of axenically cultured promastigotes is the release of vast amounts of LPG from the promastigote surface by a process that is dependent on extracellular proteins such as BSA or ovalbumin [5,35]. To date no adequate mechanism has been presented for the partitioning of LPG from the parasite membrane into the bulk solution. It is clear, however, that the mechanism appears to be specific to LPG, as the other major glycosylphosphatidylinositol-anchored molecule, gp63, is not detected in the culture medium. This selectivity may be in part attributed to the preference of lysophospholipids, such as LPG, to form micelles rather than bilayers [36]. The consistent appearance of the previously mentioned IVP during KMP-11 purification suggests that KMP-11 may be specifically hydrolysed in the loop section of the protein by gp63. As isolation is initiated with highly denaturing solvent-extraction conditions known to destroy gp63 enzymic activity, the proposed cleavage could not have transpired during purification. This proteolytic event could result in a decreased helical co-operativity similar to that already described for the apolipoprotein model [29], thereby facilitating the partitioning of LPG from the parasite plasma membrane. Such a function would offer a novel mechanism for alteration of the physicochemical properties of *Leishmania* parasite membranes, facilitating a selective change in the surface LPG which has been correlated with increased pathogenesis [33].

Finally, endonuclease restriction and Southern-blot analysis of genomic DNA indicated that the KMP-11 gene locus consists of three gene copies separated by variable intergenic regions. KMP-11 is estimated to be present at 1×10^6–2×10^6 copies per cell on *L. donovani* promastigotes, a number comparable with that reported for both LPG [30] and the major surface glycoprotein, gp63, which has been found to be encoded by as many as seven gene copies [37]. Although further investigation of the transcriptional regulation of KMP-11 in both the promastigote and amastigote forms is required, an obvious explanation for the multiple gene copies is that it provides a mechanism for high-level protein expression. Since this molecule has been detected in all kinetoplastids examined to date (C. Stebeck, R. P. Beecroft, B. N. Singh, A. Jardim, R. W. Olafson, K. D. Prenevost and T. W. Pearson, unpublished work) and is present in all *Leishmania* life-cycle stages, it is evidently a molecule of considerable significance to protozoan parasites. Further investigations are therefore warranted to clarify the cellular function of this molecule.

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