Isolation and structural characterization of the *Leishmania donovani* kinetoplastid membrane protein-11, a major immunoreactive membrane glycoprotein

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A novel membrane molecule, previously observed to be co-isolated with lipophosphoglycan and called lipophosphoglycan-associated protein, has been detected in *Leishmania donovani* promastigotes and amastigotes. This kinetoplastid membrane protein (KMP-11) has been purified by preparative SDS/PAGE after organic solvent extraction of promastigote membranes. Isoelectric-focusing experiments indicated that this was an acidic protein with an isoelectric point of 4.8. Immunoblot analysis of subcellular fractions, together with ^{125}I-labelling experiments, showed this molecule to be associated with the promastigote cell surface membrane. KMP-11 was expressed at a copy number similar to that of lipophosphoglycan (1×10^6–2×10^6 molecules per cell), making this glycoprotein one of the major features on the parasite cell surface. The primary structure, less a blocked N-terminal region, was determined by automated Edman degradation of peptides derived from CNBr or enzymic fragmentation. Several post-translational modifications were also found during these studies, including an O-linked oligosaccharide and an N^G-monomethylarginine functionality which was verified by m.s. Finally, a set of sequential synthetic peptides was made based on the established partial sequence allowing structural determination of two distinct antibody-binding sites for the monoclonal antibodies L98 and L157.

INTRODUCTION

Studies in this laboratory have indicated that the major surface lipophosphoglycan (LPG) of *Leishmania* protozoa is invariably isolated from the parasite cell membrane in association with a tightly bound protein [1]. This LPG-associated protein or kinetoplastid membrane protein-11 (KMP-11) has taken on some importance as the most likely candidate molecule responsible for the immunological activity of LPG. Earlier work had clearly shown that LPG was immunoprotective in mice [2–4] and could stimulate proliferation of peripheral blood lymphocytes from human leishmaniasis patients [4–6]. We demonstrated that the KMP-11 and not the associated LPG was responsible for this T-cell-mediated activity [1]. Although findings provided strong evidence in favour of the immunological involvement of KMP-11, essentially nothing was known about either the structure or physiological function of this unusual molecule. The present communication shows that KMP-11 is a dominant surface membrane glycoprotein found on both promastigote and amastigote forms of *Leishmania* parasites. Aside from a small N-terminally blocked N-terminal region, the amino acid sequence of the protein was determined by Edman degradation, revealing the position of an *N*-methylarginine post-translational modification and the structures of two monoclonal antibody (mAb)-binding epitopes. The following paper [7] provides further details about the translated gene sequence and gene structure, together with a predicted secondary structure and evidence in support of a hypothetical physiological function for KMP-11.

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 at 26 °C in M199 media supplemented with 1000 units/ml penicillin, 50 μg/ml streptomycin, 5 mg/l haemin (all from Sigma Chemical Co., St. Louis, MO, U.S.A.), Eagle basal medium vitamin solution (Gibco, Grand Island, NY, U.S.A.) and 5% heat-inactivated fetal calf serum (Hyclone, Logan, UT, U.S.A.). Late-exponential- to stationary-phase cultures were harvested by centrifugation at 2000 *g* and washed twice with PBS. Cells were used immediately for isolation of KMP-11 or stored at −20 °C as a cell pellet. *L. donovani* LV9 amastigotes were donated by Dr. J. Blackwell (Cambridge University, Cambridge, U.K.) and maintained in Syrian golden hamsters by injection passage of 2×10^8 amastigotes approx. every 2 months. Amastigotes for isolation of KMP-11 were harvested from infected hamster spleens by the method of Channon et al. [8].

Monoclonal antibodies

MAbs L98 and L157, developed to *L. donovani* KMP-11 [1,9], along with the anti-(human transferrin) mAb used as a negative control were gifts from Dr. T. W. Pearson (University of Victoria).

Purification of KMP-11 from *L. donovani* promastigotes

The LPG–KMP-11 complex was extracted from pelleted promastigotes or amastigotes and purified by gel permeation chromatography [10,11]. Briefly, 2×10^{11} parasites were delipidated by sequential extraction with 3×25 ml of chloroform/methanol/water (3:2:1, by vol.), 3×25 ml of chloroform/methanol/water (1:1:0.3, by vol.) and 3×25 ml of 4 mM

Abbreviations used: LPG, lipophosphoglycan; KMP-11, kinetoplastid membrane protein-11; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulphonic acid; mAb, monoclonal antibody; IVP, *in vitro* processed protein.

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$MgCl_2$. Finally, the LPG–KMP-11 complex was extracted with 4 × 25 ml of solvent E [water/ethanol/diethyl ether/pyridine/NH_4OH (15:15:5:1:0.017, by vol.). The residue was applied to a Sephadex G-100 column (2.7 cm × 34 cm), developed with 40 mM NH_4OH/1 mM EDTA and monitored by indirect e.l.i.s.a. with mAbs CA7AE and L98 [9]. The LPG–KMP-11 complex was resuspended in 4 ml of solvent E and precipitated with an equal volume of methanol for 16 h at −20 °C to remove trace levels of phospholipid. LPG was depleted from these preparations by depolymerization with 40 mM trifluoroacetic acid (TFA) and the protein component was precipitated with 20 vol. of cold acetone. The crude KMP-11 was dissolved in Laemmli sample buffer [12] and fractionated at a constant voltage (20 V/cm) on a 2.5 mm × 100 mm 7% denaturing tube gel on an Applied Biosystems model 230 preparative gel electrophoresis system (Applied Biosystems, Foster City, CA, U.S.A.), collecting 150 μl fractions. The purity of each fraction was assessed by SDS/PAGE on a 15% gel stained with Coomassie Blue and by Western blotting with mAb L98. Fractions containing a homogeneous 11 kDa immunopositive protein were pooled, dialysed, concentrated and precipitated with acetone to remove SDS.

E.l.i.s.a.

Either crude purified KMP-11 or control human transferrin was coated on to a microtitre plate (Costar, Cambridge, MA, U.S.A.) at 1 μg/well for indirect e.l.i.s.a. [13]. Ascites fluid containing either mAb L98 or L157 was used at a dilution of 1:1000 [1] and the second antibody horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (Pierce Chemical Company, Rockford, IL, U.S.A.) was used at 1:2000 dilution. E.l.i.s.a.s were developed with the substrate 2,2′-azinobis-(3-ethylbenzthiazoline)sulphonic acid (Sigma).

KMP-11 immunoblots

Solvent E extracts of *L. donovani* promastigotes [11] or purified KMP-11 were separated by SDS/PAGE and transferred to Problot membrane (Applied Biosystems) in Tris/glycine buffer, pH 8.2, at 90 V for 20 min [14], and free sites on the membrane were blocked with 10% calf serum in PBS with 0.5% Tween 20 (Sigma) at 37 °C for 1 h. Blots were probed with either mAb L98 or anti-(human transferrin) mAb as a control (1:1000 dilution), and bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse IgG/IgM using Nitroblue tetrazolium chloride 5-bromo-4-chloro-3-indoxyl phosphate *p*-toluidine salt substrate (Sigma).

CNBr digest of KMP-11

Purified KMP-11 (100 μg) was dissolved in 200 μl of 70% TFA containing 20 mg of CNBr (Eastman Kodak, Rochester, NY, U.S.A.), nitrogen sparged and incubated at room temperature in the dark with stirring for 36 h. The reaction was terminated by diluting the digest mixture with 3 ml of water and evaporating it to dryness in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). CNBr peptides were dissolved in 100 μl of 0.1% TFA and chromatographed on a PRP-1 reversed-phase h.p.l.c. column (Hamilton, Reno, NV, U.S.A.) equilibrated with 0.1% TFA and developed with a linear gradient of acetonitrile at a rate of 0.5%/min. Fractions were collected in 1.5 ml Eppendorf tubes and dried.

Proteolytic digests of KMP-11

Endoproteinase Lys-C

Purified KMP-11 (100 μg) was dissolved in 150 mM ammonium acetate/4 M urea, pH 8.0, and boiled for 5 min to aid denaturation. After cooling, 2 μg of endoproteinase Lys-C (Boehringer-Mannheim, Laval, Que., Canada) was added and the digest was incubated at 37 °C for 8 h. Peptides were separated on an Aquapore RP-300 reversed-phase column equilibrated with 0.1% TFA and developed with a linear gradient of 0.1% TFA in acetonitrile at a rate of 0.5%/min. Fractions were collected in 1.5 ml Eppendorf tubes and dried.

Endoproteinase Asp-N

KMP-11 (50 μg) was dissolved in 100 μl of 100 mM ammonium acetate, pH 8.0/1 M urea and digested overnight with 1 μg of endoproteinase Asp-N. The digest mixture was separated by reversed-phase chromatography on an Aquapore RP-300 reversed-phase column as described above for the Lys-C digests.

Peptide microsequencing

CNBr- or protease-derived peptides were dissolved in 20 μl of 20% methanol and applied to 4 mm^2 sections of Problot membrane. Membranes were washed twice with 500 μl of water, air-dried and either sequenced directly on a model 473 pulsed-liquid sequenator (Applied Biosystems) or stored at −20 °C.

Amino acid analysis

KMP-11 was dried as a film in 4 × 25 mm borosilicate tubes and subjected to gas-phase hydrolysis using 6 M HCl for 1 h at 165 °C under an argon atmosphere. Hydrolysates were dissolved in 20 μl of water and analysed on an Applied Biosystems 420 amino acid analyser.

Monosaccharide composition of KMP-11

KMP-11 (20 μg), dissolved in 200 μl of 2 M HCl, was incubated at 100 °C for 4 h in a 1.5 ml screw-top polypropylene tube. Hydrolysates were thoroughly dried in a Speed Vac concentrator to remove HCl and subsequently dissolved in 25 μl of water. The monosaccharide composition was determined on a carbohydrate-dedicated Dionex Bio-LC system equipped with a CarboPacA-1 column (Dionex Corp., Sunnyvale, CA, U.S.A.) and developed isocratically with 16 mM NaOH. Sugars were detected with a pulsed amperometric detector using the following settings: $E_1 = 0.05$ V ($t_1 = 300$ ms); $E_2 = 0.6$ V ($t_2 = 120$ ms); $E_3 = 0.6$ V ($t_3 = 60$ ms).

Mass spectrometry analysis of KMP-11 CNBr-2 peptide

The parent ion of the CNBr-2 peptide was determined by continuous-flow fast-atom bombardment m.s. on a Finnigan MAT-90 high-field mass spectrometer. The peptide was dissolved in 0.1% TFA containing 5% glycerol and injected at a flow rate of 5 μl/min.

Peptide synthesis

A 60-amino acid peptide corresponding to the KMP-11 sequence encompassing residues 32–92 was synthesized on an Applied

Biosystems 430A automated peptide synthesizer using Merrifield solid-phase chemistry. Free acid peptides were synthesized on a Wang-lysine resin using Fmoc-protected amino acids activated *in situ* with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate according to the suppliers' protocols. All solvents and reagents were purchased from Applied Biosystems except for amino acids and resins, which were obtained from Novabiochem (La Jolla, CA, U.S.A.). Synthesis of the growing peptide was interrupted twice to remove resin samples containing incomplete peptides 71–92 and 45–92. Peptides were cleaved and deprotected with trifluoromethanesulphonic acid (TFMSA) [15], purified to greater than 95% homogeneity by reversed-phase chromatography and analysed for amino acid composition.

Determination of the KMP-11 isoelectric point

Crude promastigote KMP-11 (200 μg) was depleted of LPG as indicated above, dissolved in 60 ml of 1% ampholyte (Bio-Rad, Richmond, CA, U.S.A.) solution, pH 3–10, and focused on a Bio-Rad Rotofor at 12 W for 5 h. Fractions (3 volumes) were collected and assayed for KMP-11 by e.l.i.s.a. with mAb L98. The isoelectric point of KMP-11 was determined by measuring the pH of each fraction.

Preparation of promastigote plasma membrane

L. donovani promastigote plasma-membrane and cytoplasmic fractions were prepared by differential centrifugation of cell homogenates followed by sucrose-density-gradient centrifugation of the crude membrane fraction by the method of Gottlieb and Dwyer [16]. The pellicular membrane preparation was verified by determination of LPG and membrane acid phosphatase activity [16].

Immunoprecipitation of ^{125}I-labelled promastigotes

Mid-exponential-phase promastigotes (5×10^7) were suspended in 500 μl of cold PBS/glucose and radiolabelled with 500 μCi of ^{125}I (Dupont Canada Inc., Toronto, Ont., Canada) in a borosilicate tube coated with Iodogen (Sigma) as previously described [17]. Cells were lysed with 300 μl of 1% Nonidet P40 in PBS. Immunoprecipitation was affected by adding 10 μl of either mAb L98 or anti-(human transferrin) ascites fluid to 150 μl of lysate and incubated at room temperature for 1 h. Antigen–antibody complexes were precipitated with Protein G–Sepharose.

RESULTS

Purification of KMP-11 and estimation of cell copy number

The LPG–KMP-11 complex was obtained from whole cells by extraction of delipidated promastigotes with the polar solvent E system as previously described [10,11]. The extracted complex was eluted in the void volume fractions of a Sephadex G-100 gel-permeation column, suggesting a complex molecular mass greater than 150 kDa. Coomassie Blue-stained gels of these fractions showed a major protein species eluted as a broad band (Figure 1a, lane 2) which was degraded on exposure to mild acid to a species with an approximate molecular mass of 11 kDa (Figure 1a, lane 1). This protein component, which was co-purified with LPG, was previously undetected as it migrated ahead of the dye front on 10% polyacrylamide gels [10,11]. We have previously reported that KMP-11 was purified by reversed phase chromatography from a complex containing the glycoconjugate LPG [1]. Analysis of this material by SDS/PAGE showed a series of Coomassie Blue-staining bands ranging from 11 to 40 kDa, all

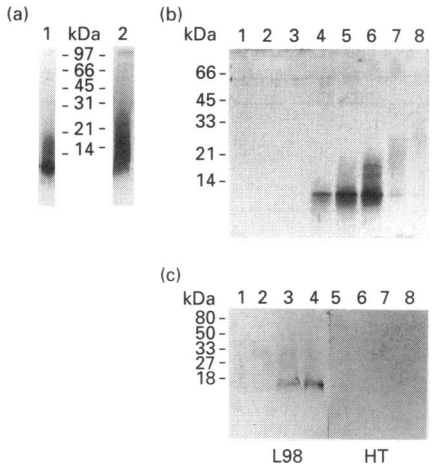

Figure 1 Purification of *L. donovani* KMP-11

(**a**) Denaturing SDS/PAGE of *L. donovani* promastigote KMP-11. Lane 1, crude KMP-11 depleted of LPG by mild acid hydrolysis; lane 2, crude KMP-11 extracted with solvent E. (**b**) Coomassie Blue-stained gel of preparative electrophoresis fractions 1–9 (lanes 1–9). (**c**) Western-blot analysis of preparative gel fractions with KMP-11-specific mAb L98 and human transferrin-specific antibody (HT; negative control): lanes 1 and 5, fraction 9; lanes 2 and 6, fraction 8; lanes 3 and 7, fraction 7; lanes 4 and 8, fraction 6.

immunoreactive with the KMP-11-specific mAbs L157 and L98 [1]. In the present communication a highly resolving preparative electrophoresis procedure was used to purify the major 11 kDa component to homogeneity for structural characterization. Before electrophoresis, the sample was subjected to mild acid hydrolysis with TFA followed by extraction of the precipitate with acetone [11]. Treatment with TFA would be expected to depolymerize the phosphodiester-linked phosphoglycans found in LPG and had no detrimental effect on the protein backbone, as judged by amino acid composition of Western-blotted samples (results not shown). Figure 1(b) shows that the preparative gel electrophoresis fractions 5 and 6 contained a single Coomassie Blue-staining protein which reacted with the KMP-11-specific mAb L98 [1] after immunoblotting (Figure 1c). Isoelectric-focusing experiments using LPG-free protein indicated that KMP-11 had an acidic isoelectric point near 4.8 (results not shown).

During the above isolation experiments it was revealed that *L. donovani* promastigote KMP-11 accounted for a significant fraction of the total plasma-membrane protein. Estimates of KMP-11, based on both gravimetric and amino acid analyses of membrane preparations, indicated a copy number of 1×10^6–2×10^6 molecules per cell, which is comparable with levels of LPG [10] and renders KMP-11 one of the major species on the parasite surface.

Subcellular distribution of KMP-11 on *Leishmania* parasites and life-cycle-stage expression

The co-isolation of LPG and KMP-11 as a stable complex suggested a close relationship between those two molecules on the parasite cell surface. However, the possibility existed that the strong binding of these two molecules was an artifact of the preparation and that the protein was actually derived from an intracellular compartment. Cellular fractionation of promastigotes showed KMP-11 to be predominantly distributed in the plasma membrane, along with the cell surface marker LPG [18]

Table 1 Subcellular distribution of KMP-11 in *L. donovani* promastigotes

LPG and KMP-11 were detected by e.l.i.s.a. with mAbs CA7AE and L98 respectively [1,9]. Promastigotes were fractionated by the method of Gottlieb and Dwyer [16].

	A_{405}	
	LPG	KMP-11
Cytoplasmic fraction	0.25	0.08
Membrane fraction	0.67	0.44

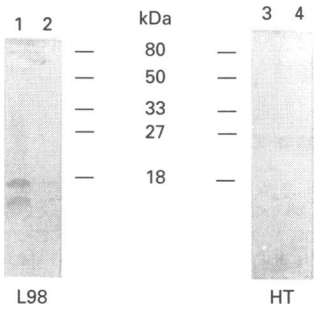

Figure 2 Western blot of *L. donovani* subcellular fractions

Lanes 1 and 3, plasma membrane purified by density-gradient centrifugation; lanes 2 and 4, 100000 ***g*** cytoplasmic fraction. An equivalent of 2×10^6 promastigotes was loaded in each lane. Identical Western blots were probed with either the KMP-11-specific mAb L98 (L98) or the control mAb specific for human transferrin (HT).

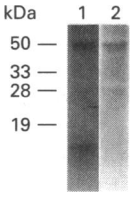

Figure 3 Autoradiography of ^{125}I-labelled immunoprecipitated KMP-11

Promastigote lysates were allowed to react with either the KMP-11-specific mAb L157 (lane 1) or the control mAb specific for human transferrin (lane 2) and the antibody–antigen complex precipitated with Protein G–Sepharose. The band observed at approximately 50 kDa in both control and experimental lanes is immunoglobulin heavy chain arising from fetal calf serum in the growth medium [32].

(Table 1). Yet neither mAb L98 nor L157 recognized KMP-11 on whole- or broken-cell preparations. Treatment of purified membranes with either 10 mM HCl or 1 M NaCl, conditions commonly used to solubilize peripheral-membrane proteins, released only trace levels of KMP-11 and suggested that the KMP-11 association with the membrane was hydrophobic in character. Immunoblot analysis of the *L. donovani* plasma-membrane and cytoplasmic fractions with L98 showed that KMP-11 was present only in the membrane fraction (Figure 2). The presence of more than one species is consistent with the polydisperse nature of KMP-11, even in the presence of SDS. The strongest evidence for surface-membrane association, however, was the demonstration that radiolabelled KMP-11 could be immunoprecipitated from ^{125}I-labelled promastigotes with mAb L157 (Figure 3).

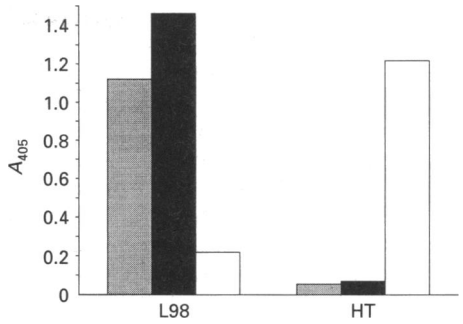

Figure 4 E.l.i.s.a. screening of *L. donovani* amastigotes for the expression of KMP-11 with mAb L98

Wells were coated with 5 μg of promastigote (■) or amastigote (▨) KMP-11. □, Human transferrin. Values are the means of triplicate assays. L98 and HT are explained in the legend to Figure 2.

A related issue pertained to the distribution of the glycoprotein on the life-cycle stages of the parasite. Figure 4 summarizes e.l.i.s.a. results using mAb L98 which show that KMP-11 is not only expressed on promastigotes but that solvent E-extractable KMP-11 is also present on *L. donovani* amastigotes. Whether amastigote levels are comparable with those found on promastigotes has yet to be determined. Thus KMP-11 emerges as a cell-surface glycoprotein expressed across *Leishmania* life-cycle stages.

Glycosylation of KMP-11

Ion-exchange chromatography of acid-hydrolysed KMP-11 on a Dionex Bio-LC system indicated the presence of galactosamine, galactose, glucose and mannose residues in equimolar amounts in the glycoprotein structure, and amino acid analysis showed that there was a 1:1 ratio of galactose to the protein. Moreover, it was significant that only galactosamine could be detected after acid hydrolysis of KMP-11 previously treated with anhydrous TFMSA. TFMSA readily cleaves all glycosidic linkages, save that between the terminal sugar and the protein, where N-linked sugars are stable and O-linked GalNAc is known to be released very slowly [20]. These findings implicated galactosamine in the attachment, probably in the form of an O-linked GalNAc. This conclusion was given further support by the absence of both a consensus sequence for N-linked sugars [7] and the absence of glucosamine found in the compositional analysis. Preliminary Bio-gel P-4 chromatography results revealed that the hydrazine-released oligosaccharide was eluted with the same hydrodynamic volume as a polyglucose tetrasaccharide standard [21]. These data are consistent with the conclusion that KMP-11 is a surface membrane molecule, as, although intracellular glycoproteins are known to occur in eukaryotes, glycosylation is a typical post-translational modification of cell surface proteins.

Primary-structure analysis and determination of a modified arginine residue

Several attempts to obtain sequence data directly from SDS/PAGE-purified KMP-11 were unsuccessful because of a modification at the N-terminus. CNBr cleavage of KMP-11 yielded four peptides resulting from cleavage at methionine residues 44, 54 and 66 (Figure 5). Using microsequencing data verified by amino-acid-composition analysis (Table 2), it was possible to

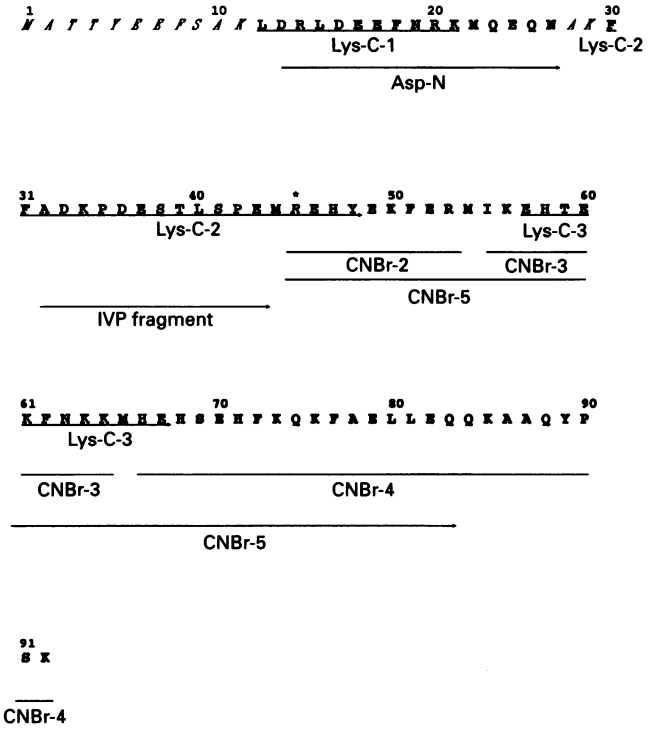

Figure 5 Protein sequence of KMP-11 isolated from *L. donovani* promastigotes

Peptides sequenced by Edman degradation chemistry are underlined. CNBr denotes CNBr fragments and Lys-C and Asp-N indicates peptides generated by the respective endoproteinase digests. The asterisk denotes the position of the N^G-monomethylated arginine residue and arrows indicate incompletely sequenced peptides. Italicized residues were determined by DNA sequencing [7]. IVP, *in vitro* processed protein.

Table 2 Amino acid composition of intact *L. donovani* KMP-11 and CNBr peptides

Values for the intact peptide are calculated residue numbers from the average of four different amino-acid-composition analyses assuming Ala = 7, based on a molecular mass of 11 kDa. Values for CNBr-1 assume His = 1.00, those for CNBr-2 assume Phe = 1.00, those for CNBr-3 assume Phe = 1.00 and those for CNBr-4 assume Ala = 3.00. Numbers in parentheses are values expected from sequence data.

	Intact	CNBr-1	CNBr-2	CNBr-3	CNBr-4
Aspartate	7.1	6.38		1.11 (1)	
Glutamate	19.7	10.4	3.38 (3)	2.23 (2)	7.83 (8)
Serine	4.8	2.62			1.82 (2)
Histidine	4.9	1.00	0.99 (1)	0.97 (1)	2.77 (3)
Arginine	3.5	4.04	1.83 (2)		
Threonine	3.6	3.60		0.97 (1)	
Alanine	7.0	4.79			3.00 (3)
Proline	3.3	0.83			0.97 (1)
Tyrosine	2.5	2.04	0.92 (1)		0.91 (1)
Methionine	4.0				
Isoleucine	1.2			0.72 (1)	
Leucine	5.1	4.28			1.95 (2)
Phenylalanine	6.4	4.71	1.00 (1)	1.00 (1)	2.04 (2)
Lysine	12.5	4.77	1.17 (1)	3.77 (4)	4.27 (4)

deduce the complete primary structure of three of these peptides, CNBr-2, CNBr-3 and CNBr-4, as shown in Figure 5. A partial CNBr digest of KMP-11 also afforded the peptide CNBr-5, encompassing residues 45–83, confirming the contiguous arrangement of peptides CNBr-2 followed by CNBr-3.

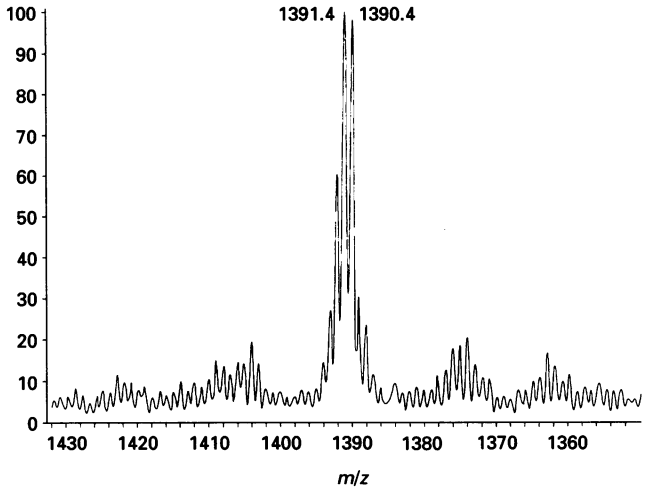

Figure 6 Continuous-flow fast-atom bombardment m.s. of CNBr-2

The observed $M+1$ parent ion was at 1391 mass units whereas that calculated from structural data was 1376 Da giving a difference of 14 mass units on subtraction of a proton. This is consistent with the modified arginine being N^G-monomethylarginine.

Interestingly, the first residue of the CNBr-2 peptide after Edman degradation did not co-elute with any of the standard phenylthiohydantoin amino acids. Amino acid analysis of this peptide indicated a composition with two arginine residues, although sequence analysis of CNBr-2 indicated only a single arginine, at position 53. Comparison of the theoretical mass of CNBr-2, calculated from the amino acid composition, with the observed parent ion determined by fast-atom bombardment m.s., showed a difference of 14 mass units (Figure 6). These data were consistent with N^G-monomethylarginine being present at position 45 in *L. donovani* KMP-11 [22].

As shown in Figure 5, endoproteinase Lys-C and Asp-N digests of intact KMP-11 provided additional information on primary structure which complemented the CNBr cleavage. More specifically, peptide Lys-C-3 completed the C-terminal region of KMP-11 by overlapping the CNBr-3 and CNBr-4 fragments. Digestion of the N-terminally blocked CNBr-1 peptide with the protease Lys-C yielded peptide Lys-C-1, which provided further information but left approximately eight residues undetermined, on the basis of amino-acid composition data. During sequence analysis of peptide Asp-N, a CNBr-resistant methionine was found, as seen at position 22 in Figure 5. This phenomenon was believed to be associated with an unusually facile oxidation to methionine sulphoxide, as subsequent cleavage attempts were successful provided that the molecule was first reduced with mercaptoethanol.

In addition to the full-length KMP-11, a truncated fragment was routinely found to be co-purified with KMP-11 on octyl-Sepharose. N-Terminal characterization of at least four different preparations gave a single protein sequence for this *in vivo* processed fragment (IVP), as shown in Figure 5. Sequence analysis suggested that, although this peptide constituted only a minor population of the KMP-11 present on the surface of the parasite, the regularity with which the sequence was detected strongly indicated a specific proteolytic event.

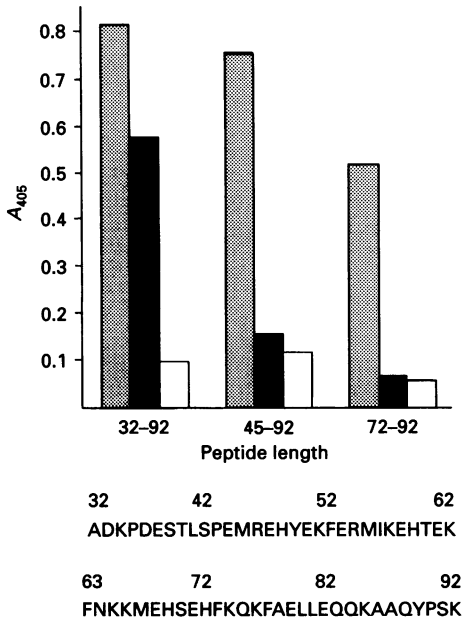

Figure 7 Epitope mapping of the KMP-11-specific mAbs L98 and L157

Peptides (10 μg/well) were dried and free sites were blocked with 100 μl of 3% skimmed-milk powder in PBS and triplicate wells were probed with mAb L98 (▨), L157 (■) or the control antibody specific for human transferrin (□).

Mapping mAb L98 and L157 epitopes

To confirm a previous finding that demonstrated that the mAbs L98 and L157 recognized protein determinants in KMP-11 rather than LPG [1], a set of nested peptides spanning residues 32–92 of the KMP-11 sequence were synthesized. Data for this study are shown in Figure 7. These results indicated that mAb L98 reacted with all three peptides, localizing the binding site to the C-terminal portion (residues 72–92) of the molecule. MAb L157, on the other hand, was found to react only with the full-length peptide, thereby narrowing its determinant to the 14 residues encompassed by amino acids 32–45, located near the centre of the proposed helix–coil–helix motif discussed in the companion paper [7].

DISCUSSION

In a previous paper [1] we reported the isolation of a potent T-cell-stimulating protein complex which was tightly associated with *L. donovani* LPG. The dominant protein component in these preparations was a heterodisperse 11 kDa species which was recognized by mAbs L98 and L157 [1,9]. In addition, several minor Coomasie Blue-staining bands of higher molecular mass also cross-reacted with these antibodies, suggesting a common larger precursor protein. However, characterization of the *kmp-11* gene, discussed in the following paper [7], clearly shows that KMP-11 is encoded by a 276 bp open reading frame which corresponds to an 11 kDa protein.

It is clear from our results that KMP-11 is found either as a highly post-translationally modified set of species or as a series of aggregates that are not readily disrupted by SDS, resulting in a ladder of molecules of various molecular mass all cross-reacting with mAbs L98 and L157. The polydisperse behaviour of KMP-11 on SDS/PAGE resulted in this dominant protein being overlooked until recently. Although initial studies of KMP-11 focused on the most abundant 11 kDa species, it was found that treatment of the LPG–KMP-11 complex with mild acid resulted in collapse of the antibody-reactive species into a more discrete single band. Although this finding appears to support the aggregation explanation for polydisperse behaviour, it remains possible that KMP-11 has covalent phosphoglycan repeats which are similar to, but not identical with, those found on LPG. [KMP-11 was not recognized by the anti-(LPG repeat) mAb CA7AE.] TFA treatment of KMP-11 has greatly facilitated purification of the protein by preparative electrophoresis and is the basis of the present isolation method.

Numerous attempts to obtain N-terminal sequence information from intact KMP-11 were unsuccessful because of a modification at the N-terminus. Consequently, the complete structure could not be ascertained by Edman degradation. The chemical nature and origin of this blocking moiety remains unknown, but is the subject of an ongoing investigation. However, elucidation of approx. 80% of the internal sequence was facilitated using chemical and proteolytic fragmentation. In addition to the 11 kDa protein, a minor proteolytic fragment (IVP) was consistently present in different KMP-11 preparations isolated from *L. donovani* promastigotes. Sequence analysis showed that this fragment corresponded to the KMP-11 C-terminal, including residues 32–92. The specificity of cleavage of the KMP-11 parent molecule, together with its position on the cell surface, makes the glycosylphosphatidylinositol-anchored metalloprotease gp63 [24] a likely candidate for this proteolytic event. Bouvier et al. [25] demonstrated that gp63 recognizes cleavage sites that contain a hydrophobe at position P1′ and basic residues at positions P2′ and/or P3′. In addition, a preference for bulky residues, such as tyrosine, at P1 was also exhibited. The observed cleavage site between residues 31 and 32 conforms to this motif with Ala-32 at P1′, Lys-34 at P3′ and the bulky Phe-31 at P1. Furthermore, the Garnier algorithm [26] predicted the above sequence to 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 or merely an interesting coincidence is a subject of investigation.

Although the protein backbone remained faithful to the gene sequence [7], KMP-11 did undergo post-translational modification involving methylation of Arg-45 to N^G-monomethylarginine. The exact role of this modification is unclear as the charge character of the side chain is retained. One possible benefit could be an increase in the hydrophobic moment allowing the arginine to be more thermodynamically stable in a non-polar environment. Monomethylated and dimethylated arginines have been detected in a number of eukaryotic proteins including histones, actin, myosin and ribosomal proteins [27] but the biological relevance of this modified residue remains to be established. Recently, Lawrence and Robert-Gero [28], using S-[^{3}H-*methyl*]adenosylmethionine as a label, demonstrated that one-third of all methylated proteins in *L. donovani* promastigotes were base-stable N-methylations of membrane-associated proteins. Moreover, they showed that the highly infectious stationary-phase promastigotes exhibited elevated methylation activity, with the *N*-methyl proteins displaying the most dramatic increases. However, no indication was made as to whether or not these membrane proteins were oriented on intracellular or extracellular surfaces.

A possible role for the monomethylarginine residues described here for KMP-11 relates to the survival of *Leishmania* parasites within the phagolysosome. Reports by Liew et al. [29] and Roach et al. [30] have implicated nitric oxide as the central leishmaniacidal agent in activated murine macrophage. Treatment of macrophage with monomethylarginine, a competitive inhibitor

of the nitric oxide synthase, resulted in inhibition of the leishmaniacidal effect allowing parasites to proliferate intracellularly. It is conceivable that in the acidic phagolysosomal environment, with a pH equal to the isoelectric point of KMP-11, the protein is released from the parasite surface and degraded in the proteolytic environment, generating free amino acids, including the inhibitory monomethylarginine. Experiments are at present underway designed to elucidate the inhibitory potential of monomethylarginine-containing peptides on nitric oxide synthase.

In addition to methylation, glycosylation was also detected on KMP-11. The absence of an Asn-X-Ser/Thr N-linked glycosylation site, together with the lack of glucosamine and presence of galactosamine in the composition, suggested that this sugar was covalently attached via an O-linked structure. Further evidence that this was the case was obtained by showing that the only sugar found after treatment of KMP-11 with anhydrous TFMSA was galactosamine, as expected for an O-linked glycoprotein [20]. Although the precise position of glycosylation has not been formally demonstrated, potential attachment sites include Thr-3, Thr-4 and Ser-9 which, because of a blocked N-terminus, are the only hydroxylated residues not sequenced by Edman degradation. To date no cell surface proteins with O-linked sugars have been reported for *Leishmania* promastigotes.

KMP-11 was estimated, by both gravimetric and amino-acid-analytical methods, to be present at 1×10^6–2×10^6 copies per cell on *L. donovani* LD3 promastigotes. The biological significance of such a predominant molecule appears to be further emphasized by its expression in a wide range of kinetoplastids (C. Stebeck, R. P. Beecroft, B. N. Singh, A. Jardim, R. W. Olafson, K. D. Prenevost and T. W. Pearson, unpublished work; [31]) and by its expression in all life-cycle stages of Leishmania parasites. The fact that KMP-11 is found in *L. donovani* amastigotes may indicate that the protein expression is not dependent on the co-expression of LPG, as amastigotes do not appear to elaborate the LPG. However, it should be noted that the relative abundance of amastigote KMP-11 is not known at present and may also be down-regulated. Nonetheless, if conservation of structure reveals functionally significant molecules, KMP-11 may prove to be a glycoprotein of substantial importance to the parasite and well worth continued effort to clarify its biological role. The protein is known to play a role in the immunology of leishmaniasis and has been implicated in immunoprotection experiments as a potential vaccine candidate [3,5]. The data reported here should provide further impetus to both areas of study.

We acknowledge the excellent technical assistance of Ms. N. Burgess in maintenance of cell cultures and of Ms. S. Kielland and Mr. D. Hardie of the Tripartite Microanalytical Center for protein sequencing and amino acid analysis. We also thank Mr. A. Labossiere and Mr. S. Scholz for their assistance with the Figures. This work was jointly supported by the B.C. Health Research Foundation and Natural Sciences and Engineering Research Council of Canada.

Received 16 May 1994/11 August 1994; accepted 17 August 1994

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