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Short Communication

Characterization of a glycosylphosphatidylinositol membrane protein anchor precursor in *Leishmania mexicana*

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A complex array of glycoconjugates, many of which are linked to the membrane by glycosylphosphatidylinositol (GPI), is expressed on the cell surface of *Leishmania* species. In the best studied *Leishmania* species, *L. major*, GPIs have been found as protein (gp63 [1,2]) and polysaccharide (lipophosphoglycan (LPG) [3,4]) anchors and as smaller glycolipids (GIPLs [5,6]). The GPI anchor of gp63 contains the consensus EthN-P-Manα1-2Manα1-6Manα1-4GlcNα1-6-inositol core, with an alkyl-acyl lipid structure [2], whilst the GIPLs are structurally divergent after the first mannose, and more closely resemble LPG precursors [5]. In *Trypanosoma brucei*, we and others have identified ethanolamine phosphate-containing GPIs that are structurally homologous to the respective protein GPI anchors [7–13], whilst such structures have not been reported in *Leishmania*. We now report the identification of a lipid with the expected structure of a GPI protein anchor precursor.

L. mexicana promastigotes were grown in 5 ml of glucose-free RPMI 1640 [9] at 2×10^7 parasites ml^{-1}. Cells were labeled overnight with [^{3}H]ethanolamine (3–30 Ci $mmol^{-1}$, Amersham Corp.) or [6-^{3}H]glucosamine (20–40 Ci $mmol^{-1}$) at 50 μCi ml^{-1} and then harvested. Lipids were extracted from the parasites with chloroform/methanol/water (CMW) [13] or chloroform/methanol (CM) followed by chloroform/methanol/water [14]. A total of 5.8×10^6 counts min^{-1} were incorporated into organic extractable material.

[^{3}H]ethanolamine-labeled CM extracts were analyzed by thin layer chromatography (TLC) on Silica 60 glass backed plates (Merck) using chloroform/methanol/water 10:10:3 (v/v/v) (system 1) or 10:10:2.5 (v/v/v) (system 2). TLC analysis on system 2 identified several species: the two major ones chromatographed as phospholipids (*Rf* 0.65 and 0.80, Fig. 1A). One polar species (*Rf* 0.21, 1.5% of the total radioactivity) co-chromatographed with P2, a GPI from *Trypanosoma brucei* bloodstream form [7]. This species also co-chromatographed with P2 on system 1 (*Rf* 0.42) and was designated LP-1. LP-1 was recovered in the CMW extract of a two-step extraction [14]. TLC analysis of the [^{3}H]glucosamine-labeled CMW extract showed a peak that co-chromatographed with P2 and the [^{3}H]ethanolamine-labeled LP-1, using systems 1 and 2, indicating that LP-1 contains both ethanolamine and glucosamine, two GPI components. The additional [^{3}H]glucosamine-labeled components (Fig. 1C), which were not labeled with ethanolamine, were PI-PLC sensitive (data not shown). They are probably previously described GIPLs [5] and were not investigated further.

TLC purified LP-1 was treated with either *B. thuringiensis* PI-PLC, rabbit serum (source of GPI-PLD), or nitrous acid [7]. All three

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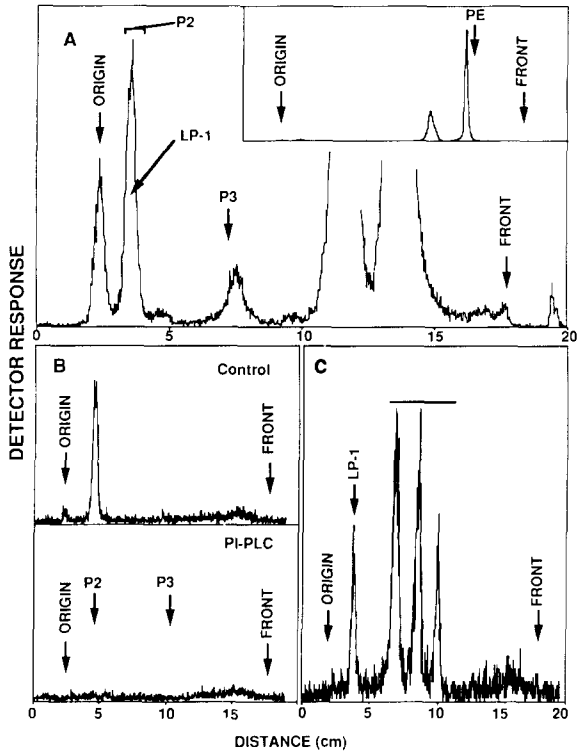

Fig. 1. Thin layer chromatographic analysis of LP-1. (A) CM extract from *L. mexicana* promastigotes labeled with [^{3}H]ethanolamine. TLC was developed using system 2. The inset shows the chromatogram at full scale. (B) PI-PLC treatment of purified LP-1. Top, control; bottom, PI-PLC. (C) CMW extract from *L. mexicana* promastigotes labeled with [^{3}H]glucosamine. The TLC was developed using system 2. P2 and P3 indicate the migration positions of co-chromatographed *T. brucei* bloodstream form GPIs. PE indicates the migration position of procyclic *T. brucei* phosphatidylethanolamine. Origin and front are indicated at 2 and 18 cm respectively. The species that chromatographed close to P3 (panel A) was not a GPI lipid based on its properties following PI-PLC and GPI-PLD digestion. Chromatograms were obtained using a Model LB2842 Berthold Linear Scanner.

treatments resulted in efficient release of the radiolabel as an aqueous-soluble fragment, suggesting that LP-1 was a GPI lipid (Fig. 1 and Table I). The remaining organic-soluble material from the deamination was found to have been converted to a less polar species on reanalysis by TLC, probably due to deamination of the ethanolamine and not the glucosamine, as has been observed for this class of lipid previously [7,9]. The aqueous-soluble

TABLE I

Sensitivity of [^{3}H]ethanolamine-labeled LP-1 to GPI-cleaving treatments

Treatment	Cleavage (%)
Nitrous acid	56.3
Control	5.7
PI-PLC	95.1
GPI-PLD	94.3
Control	1.9
Base (NH_3OH)	49.4
Control (methanol)	4.5

Nitrous acid treatment was performed at pH 3.7 and room temperature, with two additions of sodium nitrite over a 16-h period. *B. thuringiensis* PI-PLC and rabbit serum GPI-PLD digestions were carried out for 3 h at 37°C. Reactions were stopped by adding 10 μl of glacial acetic acid and immediate extraction with water-saturated butanol. Percent cleavage was estimated by the release of radiolabel into the aqueous phase of a butanol/water partitioning experiment following treatment [7]. Control reactions omitted enzyme or sodium nitrite addition. Base treatment (NH_4OH/MeOH 1:1 (v/v), 55°C, 4 h) resulted in 92.2% release of [^{3}H]mannose-labeled diacyl-PI-containing GPI species from bloodstream form *T. brucei* as aqueous-soluble material. Analysis of the base-treated LP-1 by TLC indicated that a lyso species was generated and not a free headgroup. The partial water solubility of lyso-P2 has been documented previously [7,9].

material from the nitrous acid deamination was reduced with sodium borohydride and chromatographed on a Dionex HPLC system [7]. All radioactivity coeluted EthN-P-Man_3-anhydromannitol derived from P2 and P3 [7], strongly suggesting that LP-1 contains the same headgroup as P2 and P3. Base hydrolysis of LP-1 with methanolic ammonium hydroxide produced a lyso species, based on partitioning and TLC analysis of the organic-soluble product, indicating that LP-1 contains one ether-linked glycerol substituent (Table I).

We also cultured *L. mexicana* promastigotes in the presence of mannosamine, an inhibitor of GPI synthesis [15]. We observed selective inhibition of the incorporation of [^{3}H]ethanolamine into LP-1. At a concentration of 1 mM we found that the level of radioactivity in LP-1 was decreased to 15% of the control level, but there was no reduction of the incorporation into total lipid (data not shown). This is similar to observations made in *T. brucei* and Madin-Darby canine kidney cells, except that *L.*

mexicana appears to be more susceptible to the inhibitor [15].

Several criteria indicate that LP-1 is a GPI: (i) abundance (1.5% of total [^{3}H]ethanolamine radioactivity in lipid, similar to *T. brucei* GPI precursors), (ii) labeling with [^{3}H]ethanolamine and glucosamine, (iii) co-chromatography with P2, (iv) susceptibility to cleavage with *B. thuringiensis* PI-PLC, GPI-PLD and nitrous acid, (v) co-chromatography of the nitrous acid released headgroup on Dionex HPLC with EthN-P-Man$_3$-anhydromannitol, and (vi) selective inhibition of synthesis with mannosamine. The headgroup size and the base resistance also suggest that LP-1 is structurally homologous to the gp63 GPI anchor [2] and may be its immediate precursor. Although species-specific LPG structures have been seen in *Leishmania* [5], the GPI core shows remarkable conservation and we expect that a lipid similar to LP-1 will also be present in other *Leishmania* species.

As gp63 is differentially expressed [16], it will be of interest to investigate the control of GPI precursor synthesis in different stages of the parasite life history. It is also interesting to consider how glycosylated PI is directed into the GIPL/LPG or protein anchor pathway, and if this involves compartmentalization.

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References

1 Murray, P., Spithill, T.W. and Handman, E. (1989) The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. J. Immunol. 143, 4221–4226.

2 Schneider, P., Ferguson, M.A.J., McConville, M.J., Mehlert, A., Homans, S.W. and Bordier, C. (1990) Structure of the glycosylphosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. J. Biol. Chem. 265, 16955–16964.

3 McConville, M.J., Bacic, A., Mitchell, G.F. and Handman, E. (1987) Lipophosphoglycan of *Leishmania major* that vaccinates against cutaneous leishmaniasis contains an alkylglycerophosphoinositol lipid anchor. Proc. Natl. Acad. Sci. USA 84, 8941–8945.

4 McConville, M.J., Thomas-Oates, J., Ferguson, M.A.J. and Homans, S.W. (1990) Structure of the lipophosphoglycan from *Leishmania major*. J. Biol. Chem. 265, 19611–19623.

5 McConville, M.J., Homans, S.W., Thomas-Oates, J., Dell, A. and Bacic, A. (1990) Structures of the glycoinositolphospholipids from *Leishmania major*. J. Biol. Chem. 265, 7385–7394.

6 Rosen, G., Pahlsson, P., Londner, M.V., Westerman, M.E. and Nilsson, B. (1989) Structural analysis of glycosylphosphatidylinositol antigens of *Leishmania major*. J. Biol. Chem. 264, 10457–10463.

7 Mayor, S., Menon, A.K., Ferguson, M.A.J., Dwek, R.A., Cross, G.A.M. and Rademacher, T.W. (1990) Glycolipid precursors for the membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. I. Glycan structure. J. Biol. Chem. 265, 6164–6173.

8 Mayor, S., Menon, A.K. and Cross, G.A.M. (1990) Glycolipid precursors for the membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. II. Lipid structure. J. Biol. Chem. 265, 6174–6178.

9 Field, M.C., Menon, A.K. and Cross, G.A.M. (1991) Developmental variation of glycosylphosphatidylinositol membrane anchors in *Trypanosoma brucei*. J. Biol. Chem. 266, 8392–8400.

10 Krakow, J.L., Hereld, D., Bangs, J.D., Hart, G. and Englund, P.T. (1986) Identification of a glycolipid precursor of the *Trypanosoma brucei* variant surface glycoprotein. J. Biol. Chem. 261, 12147-12153.

11 Menon, A.K., Mayor, S., Ferguson, M.A.J., Duszenko, M. and Cross, G.A.M. (1988) Candidate glycophospholipid precursor for the glycosylphosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. J. Biol. Chem. 263, 1970–1977.

12 Doering, T.L., Masterson, W.J., Englund, P.T. and Hart, G.W. (1989) Biosynthesis of the glycosylphosphatidylinositol membrane anchor of the trypanosome variant surface glycoprotein; origin of the non-acetylated glucosamine. J. Biol. Chem. 264, 11168–11173.

13 Masterson, W.J., Doering, T., Hart, G.W. and Englund, P.T. (1989) A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. Cell 56, 793–800.

14 Menon, A.K., Schwarz, R.T., Mayor, S. and Cross, G.A.M. (1990) Cell-free synthesis of glycosyl-phosphatidylinositol precursors for the glycolipid membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. J. Biol. Chem. 265, 9033–9042.

15 Lisanti, M., Field, M.C., Caras, I., Menon, A.K. and Rodriguez-Boulan, E. (1991) Mannosamine, a novel inhibitor of glycosylphosphatidylinositol incorporation into proteins. EMBO J. (in press).

16 Medina-Acosta, E., Karess, R.E., Schwartz, H. and Russell, D.G. (1989) The promastigote surface protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the amastigote stage. Mol. Biochem. Parasitol. 37, 263–274.