Identification of Complete Precursors for the Glycosylphosphatidylinositol Protein Anchors of Trypanosoma cruzi

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The survival of Trypanosoma cruzi, the causative agent of Chagas’ disease, depends vitally on proteins and glycoconjugates that mediate the parasite/host interaction. Since most of these molecules are attached to the membrane by glycosylphosphatidylinositol (GPI), alternative means of chemotherapeutic intervention might emerge from GPI biosynthesis studies. The structure of the major 1G7 antigen GPI has been fully characterized by us (Güther, M. L. S., Cardoso de Almeida, M. L., Yoshida, N., and Ferguson, M. A. J. (1992) J. Biol. Chem. 267, 6820–6828; Heise, N., Cardoso de Almeida, M. L., and Ferguson, M. A. J. (1995) Mol. Biochem. Parasitol. 70, 71–84), and based on its properties we now report the complete precursor glycolipids predicted to be transferred to the nascent protein. Migrating closely to Trypanosoma brucei glycolipid A on TLC, such species, named glycolipids A-like 1 and A-like 2, were labeled with tritiated palmitic acid, myo-inositol, glucosamine, and mannose, but surprisingly only the less polar glycolipid A-like 1 incorporated ethanolamine. The predicted products following nitrous acid deamination and digestion with phospholipases A$_2$ C, and D confirmed their GPI nature. Evidence that they may represent the anchor transferred to the 1G7 antigen came from the following analyses: (i) α-mannosidase treatments indicated that only one mannose was amenable to removal; (ii) their lipid moiety was identified as sn-1-alkyl-2-acylglycerol due to their sensitivity to phospholipase A$_2$ (PLA$_2$), mild base and by direct high performance TLC analysis of the corresponding benzoylated diradylglycerol components; and (iii) both glycolipids incorporated $^{3}$H-fatty acid only in the sn-2 and not in the sn-1-alkyl position as previously found in the GPI of the mature 1G7 antigen. Based on the differential $^{3}$H-ethanolamine incorporation pattern and the recent report that an aminoethyolphosphonic acid (AEP) replaces ethanolamine phosphate (EtNH$_2$PO$_4$) in the GPI in epimastigote sialoglycoproteins (Previo, J. O., Jones, C., Xavier, M. T., Wait, R., Travassos, L. R., Parodi, A. J., and Mendonça-Previo, L. (1995) J. Biol. Chem. 270, 7241–7250) it is proposed that glycolipid A-like 2 contains AEP and A-like 1 EtNH$_2$PO$_4$. In the in vitro cell-free system both glycolipids were synthesized simultaneously and do not seem to bear a precursor/product relationship. Among the various components synthesized in vitro a glycolipid C-like corresponding to a form of glycolipid A-like 1 acylated on the inositol was also characterized. Phenylmethylsulfonyl fluoride, an inhibitor known to block the addition of ethanolamine phosphate in T. brucei but not in mammalian cells, also inhibits the synthesis of glycolipids A-like and C-like in T. cruzi, indicating that the putative trypanosome EtNH$_2$PO$_4$/AEP transferase(s) might represent a potential target for chemotherapy.

The protozoan parasite Trypanosoma cruzi has a complex life cycle, and at all stages the majority of proteins and glycoconjugates bear a glycosylphosphatidylinositol (GPI) feature also shared by many pathogenic parasites (reviewed in Ref. 1). Since surface components are vital for T. cruzi invasion and survival within the mammalian cell (2, 3) it is firmly believed that delineation of parasite GPI metabolism might disclose leads for novel chemotherapeutic approaches. Thus, over the last years several groups including ours have been elucidating the chemical structures of these anchors (4–12), and based on them we now report the identification of candidate precursor glycolipids presumably transferred to proteins.

Metacyclogenesis, the transformation of epimastigote into metacyclic trypomastigote forms (MTCs) in the gut of the tritominate vector is a crucial step during the T. cruzi life cycle, which can be mimicked in vitro by either imposing a relative nutritional starvation (13–16) or variations in pH (17) and in temperature (18, 19). Several metacyclic-specific components, such as the 1G7 antigen and the 82-kDa and 35/50-kDa mucin-like glycoproteins, are likely to be involved in the interaction between the parasite and host cells (20–25) and are attached to...
the plasma membrane via a GPI anchor (3, 6, 26–28). Metacyclics also contain a reasonable quantity of heterogeneous GPI-anchored glycolipids known as lipopeptidophosphoglycans or LPPG molecules (29). Our group has focused on the GPI of the 1G7 antigen (1G7-Ag), a major 90-kDa metacyclic-specific antigen, which was the first T. cruzi protein to have its GPI core defined as Manα1–2Manα1–2Manα1–6Manα1–4GlcN (6). More recently the structure of its lipid moiety was shown to be essentially composed of 1-O-hexadecyl-2-octadecanoyl-β-D-phosphatidylinositol and 1-O-hexadecyl-2-hexadecanoyl-β-D-phosphatidylinositol as determined by gas chromatography mass spectrometry, phospholipase sensitivity, and high performance TLC of the benzyolated diradylglycerol components (11). The high sensitivity of electrospray mass spectrometry also revealed the unexpected presence of small quantities of inositol phosphocheramides, a component previously described only in LPPG (4, 5). Mucin-like proteins, which function as sialic acid acceptors, are released during parasite entry into mammalian cells, whereas 1G7-Ag is not (27). The recent finding that in epimastigotes the GPI of the mucin-like proteins contains alkylacylglycerol like the 1G7-Ag anchor but that in infective metacyclic these proteins bear mostly inositol phosphocheramides opens the possibility that the heterogeneous lipid portions of these two proteins might play a role in the differential release during penetration (12).

In the present paper metacyclogenesis was standardized in defined Grace’s medium, where in 72 h parasites expressed higher amounts of 1G7-Ag as compared with differentiation in nondefined liver infusion tryptose medium. Making use of metabolite incorporation of tritiated GPI components, associated to classical enzymatic and chemical analyses, two glycolipids were appointed as candidates to anchor glycoproteins in insect stages of T. cruzi. These two candidates named glycolipid A-like 1 and A-like 2, as well as a form acylated on the inositol, named glycolipid C-like, were amenable to synthesis by a crude preparation of membranes incubated with UDP-GlcNAc and GDP-[3H]mannose. Using this in vitro system it was demonstrated that PMSF is capable of inhibiting one of the final steps in the assembly of the complete precursors, as previously observed for Trypanosoma brucei (30, 31). Based on the data presently reported, a basic biosynthetic route for the multiple GPls in T. cruzi is discussed.

MATERIALS AND METHODS

Trypanosomes—Metacyclic and epimastigote forms of T. cruzi G strain (32) have been maintained alternately in mice and in liver infusion tryptose culture medium (13) or in Grace’s medium (33) in the presence or absence of 10% fetal calf serum (FCS) (Brush, Sao Paulo, Brazil) at 28°C. Epimastigotes, harvested at exponential growth in liver infusion tryptose medium were suspended in Grace’s medium at a concentration of 3 x 10⁷/ml and allowed to differentiate in the presence or absence of 10% FCS. The proportion of metacyclics was estimated by morphology and/or resistance to lysis by normal human serum as described elsewhere (34). Parasites were routinely washed once in cold 36 mM sodium phosphate buffer, pH 6.0, containing 44 mM NaCl and 300 mM glucose (PSG) following harvest. T. brucei of the Molteno Institute Trypanozoon antigenic type 1.6 (MITat 1.6) and of the ILRAD Trypanozoon antigenic type 1.21 (ILTat 1.21) were purified from citrated blood of infected albino rats by chromatography on DE-52 as described previously (35).

Preparation of Trypanosoma Lyases and Radiolabeling of Glycolipids—A Trypanosoma lyase cloning system using GDP-[3H]mannose-Radiolabeling experiments with GDP-[3H]mannose using trypanosomysy- lases were performed essentially as described by Masterson et al. (36). Briefly, bloodstream forms of MITat 1.6 T. brucei or T. cruzi obtani- ed after 1 day of culture in Grace’s medium were centrifuged at 12,000 x g for 5 min at 4°C and washed in PSG, and parasites were resuspended in 0.1 mM TPLC, 1 μg/ml of leupeptin in water at 2 x 10⁷ cells/ml. After 15 min on ice, an equal volume of 100 mM HEPES- NaOH buffer, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 0.1 mM TPLC, 1 μg/ml leupeptin, 20% glycerol (w/v) was added, and 1-ml aliquots containing 1 x 10⁶ cells were snap-frozen and stored at -70°C. At the time of use, aliquots were thawed and added to 9.0 ml of 50 mM HEPES buffer, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 0.1 mM TPLC, 1 μg/ml leupeptin on ice. After 10 min of centrifugation at 6000 rpm in a Sorvall HB-4 rotor at 4°C, the pellet was washed twice in 10 ml of ice-cold 50 mM HEPES buffer, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 0.1 mM TPLC, 1 μg/ml of leupeptin as described elsewhere (37). The membranes were suspended in 1 ml of HEPES buffer, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 0.1 mM TPLC, 1 μg/ml leupeptin. Each standard incubation mixture contained 1 x 10⁶ cell equivalents of these trypanosome membranes (100 μl) and an additional 1 ml UDP-GlcNAC, 1 mM ATP, 0.5 mM dithiothreitol, 0.2 μg/ml of tunicamycin, and 0.3 μCi of GDP-[3,4,6-3H]mannose (29.1 Ci/mmol, Amersham, Buckinghamshire, UK) or 50 μCi/ml of 1-15N ethyl 1-15N lutidine hydrochloride (28.8 Ci/mmol, Amersham) or 50 μCi/ml of myo-[3H]inositol (80–120 Ci/mmol, DuPont NEN) was dried down using a Speed Vac evaporator (Savant Instruments, Inc.). Dried palmitic acid was resuspended in 15 μl of ethanol and 1 μl of PSG containing 1 mg/ml defatted bovine serum albumin (Sigma) previous to the addition to the medium. The medium in the case of myo-inositol and ethanolamine the label was directly solubilized in myo-inositol-depleted or complete medium, respectively. The duration of each incorporation, always carried out at 28°C, is mentioned in the respective experiment, and the viability of T. cruzi checked throughout was usually greater than 95%.

Radiolabeling of T. brucei in Vivo—In order to obtain [3H]myristate-labeled glycolipid standards MITat 1.6 T. brucei bloodstream forms were labeled for 1 h at 37°C with 100 μCi/ml [3H]myristic acid (55 Ci/mmol, Amersham) in Dulbecco’s modified Eagle’s medium using the basic protocol described above for T. cruzi.

Extraction of Lipids after Radiolabeling Experiments—At the end of labeling experiments, 100 μl of trypanosomes or membrane fractions were transferred to 600 μl of chloroform/methanol (1:2, v/v) so as to obtain the proportion of 10:10:3 (v/v) of chloroform/methanol/water as described by Doering et al. (37). Samples were extracted at room temperature for 1–2 h, and insoluble material was removed by a brief centrifugation. The single-phase supernatant was dried down under a N2 stream at 37°C and resuspended in 100 μl of water-saturated 1-butanol (9:1, v/v). After the addition of water and vortexing for 30 s, the mixture was separated after a brief centrifugation, and the upper 1-butanol phase was transferred to a clean tube. The aqueous phase was reextracted with 100 μl of 1-butanol, and the combined upper phases were back-extracted once with 100 μl of 1-butanol-saturated water as described by Krakov et al. (38). The final 1-butanol phase was dried and redissolved in 15 μl of 1-butanol for fractionation on TLC or HPTLC. In the case of a large scale preparative procedure, labeled parasites were initially suspended in 1 ml of PSG and transferred to 6.9 ml of chloroform/methanol (1:1, v/v). The subsequent extractions with 1-butanol were made with twice the volumes described above.

Glycolipid A, C, and LPPG Standards—[3H]Myristate-labeled glyco- lipid A (2 and 6) or ethanolamine-PO4-Manα1–2Manα1–2Manα1–6-myo-inositol-1-P0₃(3[sn-1,2-dimyristoylglycerol]) (38, 39) and glyco- lipid C (P3 or ethanolamine-PO4-Manα1–2Manα1–2Manα1–6-GlcNα1– 6-(acyl)myo-inositol-1-PO₃(3[sn-1,2-dimyristoylglycerol]) (40–42) were purified by preparative thin layer chromatography from lipid extracts of [3H]myristate-labeled MITat 1.6 T. brucei bloodstream forms. The silver nitrate stained lipids were purified by preparative thin layer chromatography on an octyl-Sephrose 4B column from lipid extracts of [3H]palmitolabeled Trypanozoon T. cruzi epimastigote forms and was a kind gift of Dr. Rosa M. de Lederkremer (Universidade de Buenos Aires, Argentina).

Enzymatic Treatments—T. brucei phospholipase C was obtained from VSG-depleted membranes of variant ILTat 1.21 as described by Ward et al. (43). Bacillus thuringiensis Pi-specific phospholipase C was purchased from Funakoshi Ltd, Japan. Enzyme treatments were rou- tinely carried out in 50 mM Tris-HCl, 5 mM EDTA, pH 8.0, containing 0.2% Nonidet P-40 (w/v) for 3 h at 30°C for T. brucei phospholipase C.
and at 37°C for B. thuringiensis P1-specific PLC. GPI-specific phospholipase D (GP1PLD) was purified from human serum (44, 45), and digestions were carried out in 10 mM Tris acetate, pH 5.4, containing 0.1% Triton X-100. These three phospholipases were used in a 50-μl reaction mixture at 1 unit/ml as described by Cardoso de Almeida et al. (44). The PLAs, from bee venom was purchased from Boehringer Mannheim (Germany), and used as 250 units/ml dissolved in 50 μl of 100 mM Tris·HCl, 1 mM CaCl₂, pH 7.4, containing 0.05% Nonidet P-40. The α-mannosidase from jack bean (Oxford Glycosystems, UK) was used at 10 units/ml in 50 μl of 100 mM sodium acetate, 2 mM ZnCl₂, pH 4.5, for 20 h at 37°C. At the end of each reaction, it was extracted twice with 2 volumes of 1-butanol saturated with water. The pooled extracts were washed once with water saturated with 1-butanol and dried under a stream of N₂. Diradylglycerol residues produced after digestion with phospholipase C were converted to their respective benzoyl diradylglycerol derivatives and fractionated on a system discriminating diacyl-, alkacyl-, or dialkyglycerols as described by Büttiker et al. (46) and Blank et al. (47). Briefly, the products liberated from samples treated with B. thuringiensis P1-specific PLC were extracted into water-saturated 1-butanol, dried, and incubated with 100 μl of freshly prepared 3% (w/v) benzoic anhydride, 1.2% (w/v) 4-dimethylaminopyridine in benzene for 1 h at room temperature. The reaction was stopped by placing the tube on ice and slowly adding 660 μl of 0.1 M NaOH to the reaction mix. In parallel, several standards initially at 10 mg/ml in methanol were dried and benzoylated using the same protocol. These standards included diacylglycerol (DG) 1,2-di-stearoyl-glycerol, sn1,3-sn2 di-1,2-dioleoyl-glycerol; diacylglycerol (1,2-di-O-hexadecyl-rac-glycero-d), and ceramide types III and IV (Sigma, UK); a standard of alkacylglycerol (sn1-hexadecyl-2-palmitoyl-glycerol) was generated by digestion of 10 mg of α-ω-phosphatidylcholine with 5 units of phosphatidylcholine-phosphohydrolase, Type V, from Bacillus cereus (Sigma) in 20 mM Tris acetate, pH 7.4, 0.1% Triton X-100 for 3 h at 37°C, followed by extraction into hexane. Benzoyl derivatives of diradylglycerols were extracted with hexane, dried under a stream of N₂, and analyzed by HPTLC as described below.

Nitrous Acid Deamination—Dried [³H]-labeled glycolipids were resuspended in 25 μl of 0.3 M sodium acetate, pH 4.0, containing 0.02% Zwittergent 3–16 (w/v). Treatment was performed at 60°C by the addition of 25 μl of 0.5 M NaNO₂. Equal volumes of buffer and freshly made NaNO₂ solutions were added after 1 and 2 h. At the end of each reaction, the mixture was extracted with 2 volumes of water-saturated 1-butanol as described above.

Mild Base and Acid Hydrolyses—Base treatment was performed in 20 μl of 50 mM NaOH, 90% ethanol for 40 min at room temperature and terminated with 80 μl of 20% acetic acid, and the products were extracted in 1-butanol saturated with water. In this condition fatty acids linked via ester bonds to the GPI of VSG (48) and glycolipid A (38) are hydrolyzed, but fatty acids linked via other bonds, such as alkacylglycerol, or via amide bonds, as in the case of ceramides, are not (49, 50). Mild acid hydrolysis was used as a diagnostic treatment for the presence of galactose residues (Gal) as residues as described for LPPG (4). This was performed in 50 μl of 20 mM trifluoroacetic acid for 2 h at 100°C, and the reaction products were extracted in 2 volumes of water-saturated 1-butanol as described above.

Thin Layer Chromatography—Thin layer chromatography was performed on glass-backed Silica Gel 60 TLC sheets (20 cm), and high performance thin-layer chromatography was performed on aluminum plates. Silica Gel 60 HPTLC sheets (10 and 20 cm) from Merck (Darmstadt, Germany). The total lipid extracts as well as the T. brucei phospholipid C, B. thuringiensis P1-specific PLC, GPIPLD, PLA₂, α-mannosidase, mild base, mild acid, and deamination reaction products were analyzed using solvent system A (chloroform/methanol/water (10:10:3, v/v/v)) or B (benzoyl-diradylglycerol and respective benzoylated standards were analyzed on glass-backed Silica Gel 60 HPTLC sheets (10 cm) using solvent system B (benzene/hexane/diethyl ether (50:45:4, v/v/v)). The labeled glycolipids were detected by fluorography after spraying with EN³HANCE (DuPont) using XAR-5 films (Kodak, UK) and DuPont Cronex intensifying screens. The molecular mass markers (Sigma) used were myosin, 205 kDa; Escherichia coli β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa, and carbonic anhydrase, 29 kDa.

RESULTS

Defining Reproducible Metacyclogenesis Conditions Favoring Expression of High Levels of GPI-Anchored 1G7-Ag—Since 1G7-Ag is one of the major proteins in metacyclic forms and is absent in epimastigotes (20), it was reasoned that generation of the corresponding anchor precursors should precede or be concomitant with the expression of the protein itself during metacyclogenesis. Therefore, the expression of this glycoprotein was assessed in differentiation conditions using Grace’s medium in the absence or presence of 10% FCS. In the serum-enriched condition (Fig. 1A, left panel) the number of parasites increased 10-fold by day 8 in culture (black circles), and high viability (open circles) was observed up to day 12 in culture. On the other
hand, parasites cultivated in the absence of FCS (Fig. 1A, right panel) did not grow, and after 4 days viability was seriously impaired (Fig. 1A, open circles). However, under both conditions an average of about 50% of parasites, and occasionally up to 65% differentiated into MTCs, taking 4 days in the absence of serum and about 12 in its presence (Fig. 1B). Moreover, in the absence of serum much higher amounts of 1G7-Ag were expressed when compared with the parasites grown with 10% FCS (Fig. 1C). This suggested that in spite of or due to stress, parasites were able to increase the expression of 1G7-Ag, a GPI-anchored protein. Given the possibility that higher expression of a GPI-anchored protein might be accompanied by an increase in the levels of GPI precursors, coupled with the fact that metabolic incorporation of radiolabeled components into GPI precursors was markedly improved in Grace's medium depleted of their corresponding nonradioactive components, the remaining experiments were performed using the metacyclogenesis protocol in the absence of FCS.

A Candidate GPI Precursor Is Suggested by the Kinetics of [3H]Palmitate Incorporation into Lipids and Proteins during Metacyclogenesis—The profiles of [3H]palmitic acid-labeled lipids and proteins were analyzed by TLC and SDS-PAGE on day 1 and day 3 of metacyclogenesis. [3H]Palmitate-labeled proteins were observed only on day 3 and not at all on day 1, when the majority of the parasites were epimastigotes (Fig. 2). The identities of the 90-kDa, 82-kDa, and 35/50-kDa labeled proteins were ascertained by immunoprecipitation with the monoclonal antibodies 1G7, 3F6, and 10D8, whereas the presence of [3H]-fatty acid on the GPI anchor was confirmed by PLC removal (Ref. 27 and data not shown).

The complex pattern of lipids labeled over a 3-h incorporation showed an overall increase of radioactivity in the region of phosphatidylcholine (PC) and phosphatidylserine (PS) or above, both at day 1 and day 3 (Fig. 2). In contrast, a species migrating near glycolipid A of T. brucei (Fig. 2A, arrow) was intensely labeled at day 1 all over the 3 h of incorporation, whereas at day 3 it was only weakly detectable up to 10 min. The behavior of this component at day 3 was indicative of an intermediate being rapidly converted into a product absent from the TLC, thus compatible with a glycolipid being transferred to nascent proteins. Further evidence in this direction was the observation that indeed if at day 3 parasites were pulsed for 2 min with [3H]palmitic acid and chased with cold fatty acid, this glycolipid disappeared within 10 min (Fig. 2C), suggesting a rapid turnover expected from a small pool of precursor GPI. Since concomitant to this pattern of kinetic behavior, [3H]-fatty acid accumulates into the GPI-anchored glycoproteins (Fig. 2B), it was decided that this species, named glycolipid A-like (Fig. 2A, arrow), deserved further investigation.

Glycolipid A-like Is Indeed a Typical GPI with an sn-1-Alkyl-2-acylglycolipid Moiety—In order to confirm the GPI nature of the candidate glycolipid A-like, it was labeled with [3H]palmitate and myo-[3H]inositol, purified from the TLC plate, and submitted to α-mannosidase treatment as well as treatment with phospholipases C, D, and A2 as shown in Fig. 3. Several features of glycolipid A-like are compatible with the precursor properties of 1G7-Ag GPI. First, its relative migration (lanes 3 and 10, indicated by arrows) is slightly more polar than T. brucei glycolipid A, a property expected for a GPI with a glycan core presenting more than three mannoses (6). Second, the candidate species treated with α-mannosidase (αMan, lanes 2 and 9) produced a compound migrating slightly faster than glycolipid A of T. brucei, and since neither longer nor

![Fig. 2. Time course of [3H]palmitic acid incorporation into lipids and glycoproteins during metacyclogenesis: analysis of the lipid profile during a pulse-chase experiment concomitant with the expression of GPI-anchored proteins. Parasites cultivated for 1 or 3 days in Grace's medium without FCS were incubated in Grace's medium containing 0.1 mg/ml delipidated bovine serum albumin and 100 µCi/ml [3H]palmitate at 28°C. After 2 min (lanes 1), 10 min (lanes 2), 30 min (lanes 3), 60 min (lanes 4), 120 min (lanes 5), and 180 min (lanes 6) two aliquots of 3 x 10⁷ parasites were harvested. One of them was extracted in chloroform/methanol/water (10:10:3, v/v) (solvent A) followed by partition into water-saturated butanol. The other was directly solubilized into SDS-PAGE sample buffer. Panel A shows the autoradiogram of the lipid profiles of TLCs developed with solvent A. A, T. cruzi lipids labeled with [3H]myristic acid are indicated on the left of each plate; glycolipid A (Gly A), glycolipid C (Gly C), phosphatidylcholine/phosphatidylserine (PC/PS), phosphatidylethanolamine (PE), free fatty acid (FFA), and dimyristoylglycerol (DMG). Panel B shows the autoradiogram of the samples analyzed by SDS-PAGE/fluorography, and the molecular mass markers are indicated in kDa on the left. In a separate experiment parasites at day 3 of culture were submitted to a 20 min pulse with [3H]palmitate followed by 10 and 20 min of chase in the presence of 250 µM of palmitic acid. Aliquots of 3 x 10⁷ parasites were extracted in chloroform/methanol/water (10:10:3, v/v), and the lipids were resolved by TLC in the same solvent. Panel C shows the respective densitometric profiles of the TLC fluorogram, and the arrows indicate the relative position of migration of the species glycolipid A-like, putative candidate precursor of the protein GPI anchors.](image-url)
shorter digestions yielded alternative products (data not shown), these data were taken as evidence for a single terminal α-mannose in the core followed by a substituted residue, as described for the 1G7-Ag GPI (6).

The glycolipid A-like species had its GPI nature confirmed by treatments with T. brucei phospholipase C (Fig. 3, lanes 4 and 11), B. thuringiensis PI-specific PLC (lanes 5 and 12), and GPIPLD (lanes 6 and 13). As expected, in the case of [3H]palmitate-labeled species, the reaction products co-migrated with dimyristoylglycerol (DMG), free fatty acid (FFA), phosphaticid acid (PA), and glycolipids C (Gly C) and A (Gly A) are indicated. The arrows and Lyso indicate the positions of intact and lyso-species of glycolipid A-like, respectively.

The treatment with bee venom PLase apo, which specifically degrades fatty acids linked through an acyl bond to the carbon 2 of the glycerol backbone, yielded a lipid co-migrating with free fatty acid (FFA) in the case of the [3H]palmitate-labeled candidate (Fig. 3, lane 7) and the predicted sn-1-fatty acid-2-lyso-species from the myo-[3H]inositol-labeled glycolipid A-like (lane 14). The fact that the lyso-species was not detectable for the [3H]fatty acid-labeled glycolipid A-like indicated clearly that the tritiated fatty acid was just introduced in the sn-2 acyl and not in the sn-1 alkyl position, an incorporation pattern identical to that observed for mature 1G7-Ag (11).

The suspected sn-1-alkyl-2-acyl-glycerolipid nature of [3H]palmitate-labeled glycolipid A-like was confirmed by generating diradylglycerol moieties with B. thuringiensis PI-specific PLC followed by analyses of the benzoylated derivatives by HPTLC side by side with the various standards. The results indicated that 90% of the lipid product comigrated with benzoyl-alklyacylglycerol and 10% with benzoyl-ceramide standards (data not shown). However, since glycolipid A-like was not 100% pure and the proportion of benzoyl-ceramide detected was small, such a degree of heterogeneity would be better assessed by electrospray mass spectrometry, as described earlier for 1G7-Ag GPI (11).

Glycolipid A-like is actually composed of two species, of Which Only the Less Polar Is Labeled by [3H]Ethanolamine—Occasionally glycolipid A-like did not present a good resolution by TLC; therefore, several metabolic incorporations were analyzed by HPTLC. In Fig. 4 it is shown that glycolipid A-like is composed of two clearly defined bands: a less polar named A-like 1 and a more polar glycolipid A-like 2, both labeled with [3H]palmitate (lane 4) and myo-[3H]inositol (lane 7) as well as with [3H]mannose and [3H]glucosamine (data not shown). Although in [3H]fatty acid incorporations glycolipid A-like 2 appears slightly predominant over A-like 1 (lane 4), [3H]ethanolamine unexpectedly labeled just A-like 1 and not at all glycolipid A-like 2 (lane 10). Both glycolipids from T. cruzi (lanes 4–12), and [3H]myristate markers from T. brucei (lanes 1–3) were subjected to B. thuringiensis PI-specific PLC and GPIPLD treatment, and in all cases glycolipid A-like 1 and A-like 2, like T. brucei glycolipid A, were digested by these enzymes, albeit to a lesser degree with respect to GPIPLD (lanes 9 and 12). On the other hand, although glycolipid C from T. brucei was sensitive to PLD and resistant to PLC (compare lanes 3 and 2), analogous behavior was not observed for the material migrating where a glycolipid C-like molecule from T. cruzi was expected (compare lanes 5 and 6, lanes 8 and 9, and lanes 11 and 12 in the corresponding region).

Glycolipids A-like 1 and A-like 2 as Well as Other Precursors Can Be Synthesized in a Cell-free System—In order to investigate cell-free biosynthesis as devised by Masterson et al. (36) for T. brucei a similar system was established for T. cruzi.
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Washed membranes were supplemented with UDP-GlcNAc, pulsed for 15 min with GDP-\([3H]\)mannose, and chased for up to 60 min with cold GDP-mannose. The profile of synthesized products fractionated by HPTLC is shown in Fig. 5 (lanes 1–6). Both glycolipids A-like 1 and A-like 2 were synthesized in vitro and comigrated exactly with the respective in vivo \([3H]\)palmitate-labeled species (lane 9). Contrary to the in vivo incorporation of tritiated fatty acid and inositol (Fig. 4, lanes 4 and 7), the in vitro system allowed better labeling of glycolipid A-like 1 compared with A-like 2 (Fig. 5, lanes 1–6). The proportional increase in synthesis of A-like 1 and A-like 2 during the chase period (Fig. 5, lanes 2–6) indicates that one species is not precursor of the other but rather that both are terminal products of the same pathway.

In the in vitro system dolichol-phosphoryl-mannose (DPM) and several other compounds (indicated by I, II, III, VI, and VII in Fig. 5) were also labeled by \([3H]\)mannose, and despite the fact that the decrease of their respective intensities during chase appears minimal in the figure it can be easily observed in less exposed autoradiograms. This is an indication that some of the observed species are likely to be the biosynthetic precursors of glycolipids A-like 1 and A-like 2. And indeed, compounds I, III, and VI were shown to be sensitive to B. thuringiensis PI-specific PLC and GPIPLD (Fig. 5, lanes 7 and 8). Compound VII was insensitive to B. thuringiensis PI-specific PLC but sensitive to GPIPLD, a pattern consistent with a GPI containing a fatty acid esterified to inositol (56); due to previously described properties observed when synthesized in vivo (54) as well as to its peculiar sensitivity to mild base (shown below; see Fig. 7) it was named glycolipid C-like. Species IV and V were not clearly detectable in this particular experiment, but in others (data not shown and Ref. 54) they were shown to be nitrous acid- and phospholipase-insensitive and thereby considered non-GPI species, possibly attributable to insufficient tunicamycin in the reaction mixtures, as occasionally observed for the T. brucei control (data not shown).

The migration of species I, III, and IV on HPTLC, as well as their susceptibilities to phospholipases (lanes 7 and 8) and to nitrous deamination (data not shown), suggests very strongly that they correspond to the sequentially mannosylated precursors Man\(_1\)GlcN-PI, Man\(_2\)GlcN-PI, and Man\(_3\)GlcN-PI, respectively.

Comparison of the cell-free \([3H]\)mannose-labeled lipids from T. brucei (lane 10) and T. cruzi (lanes 1–5) reveals that GPI intermediates such as the remodeled species like glycolipids A’, A”, or \(\theta\) of T. brucei (55), i.e. with more polar characteristics than glycolipid A-like 1 and A-like 2, were not observed in T. cruzi. The profile of in vitro synthesized glycolipids was reproducible and independent of the presence or absence of ATP, coenzyme A, and/or palmitoyl-coenzyme A in the reaction mixtures (data not shown). Therefore, taken together these observations seem to indicate that the acyl groups of T. cruzi GPIs are not remodeled in vitro.

PMSF Inhibits GPI Anchor Biosynthesis in T. cruzi—PMSF has been shown to inhibit the addition of ethanolamine phosphate to the Man\(_2\)GlcN-PI intermediate in bloodstream form T. brucei (30). More recently, Güther et al. (31) have shown that PMSF also inhibits inositol acylation in the African trypanosome, but not in mammalian cells. Thus, the effect of PMSF on biosynthesis of GPIs in the cell-free system of T. brucei and T. cruzi was compared (Fig. 6). In the absence of PMSF, T. brucei synthesized all of the typical products (Fig. 6A), but when incubated with PMSF prior to the addition of label, the formation of glycolipids A’, A”, and \(\theta\) were reduced and there was accumulation of less glycosylated intermediates (Fig. 6B). In the case of T. cruzi although an accumulation of intermediates could not be observed in this particular figure it is clear that glycolipids C-like (VII), A-like 1 (I’), and A-like 2 (2’) were not detected in the presence of PMSF (compare panels C and D), an effect that was enhanced under conditions where the glycolipids were chased for 15 min with cold GDP-mannose (compare panels E and F).

Investigation of the Lipid Moiety of Glycolipids C-like (VII), A-like 1, and A-like 2—Since the analysis of the benzoylated lipid moiety of glycolipid A-like indicated the presence of alkylacylglycerol and ceramide (data not shown), lipid heterogeneity could be responsible for the migration difference between glycolipid A-like 1 and A-like 2. In order to test this possibility, and also to assess the type of lipid moiety of glycolipids A-like 1, A-like 2, and C-like (species VII), the corresponding \([3H]\)mannose-labeled glycolipids synthesized in vitro were purified from the HPTLC plate and treated with mild base that cleaves acyl-linked fatty acids but has no effect on alkyl- or ceramide-type linkages (50, 57). As shown in Fig. 7, glycolipids VII (lanes 1 and 2), A-like 1 (lanes 3 and 4) and A-like 2 (lanes 5 and 6) were cleaved, generating a lyso-species, thus confirming that alkylacylglycerol is present in all of them. The sharp shift after base treatment of glycolipid VII (C-like) taken together with its behavior of resistance to B. thuringiensis PI-specific PLC and
susceptibility to GPIPLD (Fig. 5, lanes 7 and 8) strongly indicates that glycolipid VII is acylated in the inositol ring. Moreover, since deacylated species VII comigrated precisely with lyso-glycolipid A-like 1 and not with lyso-A-like 2 (compare lanes 2, 4, and 6), it is reasonable to assume that glycolipid C-like is probably the inositol-acylated form of glycolipid A-like 1.

An alternative explanation for the differential migration between glycolipids A-like 1 and A-like 2 could be the presence of sn-2-O-octadecanoyl in glycolipid A-like 1 and sn-2-hexadecanoyl in glycolipid A-like 2. It has been reported that the lipid portion of the 1G7-Ag and mucin-like proteins of insect stages are composed mainly of sn-1-O-hexadecyl-2-octadecanoyl-glycerol and sn-1-O-hexadecyl-2-hexadecanoyl-glycerol, respectively (11, 12). However, as shown in Fig. 7, the migration difference between glycolipids A-like 1 and A-like 2 was also present in the corresponding lyso-species generated by base treatment, suggesting that the structural variation is not attributable to chain-length heterogeneity in the acyl-linked fatty acids. Given that the 1-O-hexadecyl-glycerol seems to be ubiquitous in all T. cruzi GPIs analyzed to date (6–8, 10, 11, 27, 58), as well as in inositol phospholipids (59), the data presented in Fig. 7 suggest strongly that the structural variation between glycolipids A-like 1 and A-like 2 must reside in a portion of the molecule other than the lipid moiety.

Comparative Properties of Glycolipids A-like 1, A-like 2, and C-like Synthesized in Vivo and In Vitro—In order to facilitate a comparison of the glycolipids characterized in this paper with LPPG and with glycolipids A and C from T. brucei, a summary of the information gathered from PLC, PLD, PLAA, mild base, jack bean α-mannosidase, and trifluoroacetic acid treatments is given in Table I. As expected, T. brucei glycolipids A and C were not affected by α-mannosidase and trifluoroacetic acid treatments, since their structures have neither unsubstituted terminal α-mannose residues in the glycan core or residues susceptible to trifluoroacetic acid such as Galα(1). On the other hand, LPPG labeled with [3H]palmitate, which was composed of three bands migrating very close to T. cruzi glycolipids A-like 1 and A-like 2, was susceptible to PLC and trifluoroacetic acid but unaffected by PLAA or mild base (Table I), as predicted for this glycoconjugate containing Gal residues and ceramide (4, 5).

Glycolipids A-like 1 and A-like 2 synthesized both in vivo and in vitro were susceptible to PLC, PLD, PLAA, and mild base treatments (Figs. 3 and 7). The last two treatments resulted in lyso-species visualized on HPTLC when glycolipids A-like 1 and A-like 2 were labeled in vivo with [3H]inositol or in vitro with [3H]mannose (Fig. 3, lane 14; Fig. 7, lanes 4 and 6; aster-
The characteristic migration of A-like 1 and A-like 2 was not altered by trifluoroacetic acid treatment, indicating the absence of Galα residues attached to their glycan cores (Table I). When assayed separately for α-mannosidase treatment, both glycolipids shifted their migration similarly, as observed in Fig. 3, but still kept their relative position in HPTLC (data not shown, Table I).

One remarkable difference concerning in vivo and in vitro biosynthesis relates to glycolipid C-like. The species synthesized in vitro and visualized by [3H]mannose incorporation presents all of the characteristics of an inositol-acylated GPI. However, the respective product labeled with [3H]palmitic acid or myo-[3H]inositol in vivo, is resistant to nitrous acid deamination and partially susceptible to PLD (54, 60), suggesting strongly that the "band" visualized by in vivo labeling is composed of multiple components including one synthesized in vitro.

**DISCUSSION**

Our aim was to characterize a glycolipid with the structural features predicted for the GPI of 1G7-Ag (6, 11), assuming that the glycolipid synthesis should be concomitant of an expression of the protein itself along metacyclogenesis (61, 62).

This end differentiation was standardized in Grace's medium, reported suitably for this purpose (63), and 50–60% of metacyclics were observed after 12 days in the presence of FCS, whereas in the absence of serum the same proportion of parasites differentiated in just 4 days (Fig. 1, A and B). Despite the fact that the viability of the parasite population was partially compromised in the absence of serum, the overexpression of 1G7-Ag (Fig. 1C) combined with the absence of serum and the completely defined medium itself favored the efficiency of radiolabeling and facilitated the present studies.

The competence of the parasite in producing the chased anchor precursor under these experimental conditions was validated by the detection of the typical GPI-anchored proteins (1G7, 3F6, and 100B antigens) (3) labeled with [3H]palmitic acid at 72 h (day 3) (Fig. 2B) but not at 24 h (day 1) of metacyclogenesis. On the other hand, the corresponding profiles of lipid showed a component with the predicted mobility for the sought anchor precursor, i.e. slightly more polar than glycolipid A of T. brucei (Fig. 2A, arrows). This species, thus named glycolipid A-like, was strongly labeled over the whole time course at day 1 but was only barely detectable at up to 10 min of incorporation on day 3 (Fig. 2A). This was the behavior predicted for the candidate which at day 3 was being actively transferred to proteins as compared to day 1. This prediction gained strength when day 3 parasites pulsed for 2 min with [3H]palmitate-labeled glycolipids and chased with cold fatty acid for 10 and 20 min showed glycolipid A-like rapidly disappearing relatively to most of the other lipids (Fig. 2C, arrow).

Glycolipid A-like was further shown to be resolved by HPTLC in two species, glycolipid A-like 1 and a slightly more polar glycolipid A-like 2 (Fig. 4). Besides palmitic acid both species incorporated tritiated myo-inositol (Fig. 4), mannosate, and glucosamine (data not shown), but quite interestingly only glycolipid A-like 1 incorporated [3H]ethanolamine (Fig. 4, lane 10). Their GPI nature was confirmed by nitrous acid deamination sensitivity (Table 1) and also by analyses of the products of enzymatic digestions with PLCs and GPIPLD (Figs. 3 and 4). The migration pattern of the α-mannosidase-treated glycolipids in a more polar position than that of the native species was compatible with the loss of a single unsubstituted nonreducing α-mannose of the core (6). Neither A-like 1 nor A-like 2 was sensitive to trifluoroacetic acid (Table I), and both PLA2 and base treatments (Figs. 3 and 7, respectively), as well as analysis of glycolipid A-like benzoylated diradylglycerols (data not shown), indicated a lipid moiety essentially composed of sn-1-alkyl-2-acylglycerol. Hence, all of these properties of glycolipid A-like 1 and A-like 2 were in perfect agreement with those reported for the anchor of 1G7-Ag (6, 11).

A T. brucei cell-free system analogous to the one described by Masterson et al. for T. brucei (36) was shown to efficiently synthesize glycolipids A-like 1 and A-like 2 in the presence of UDP-GlcNAc and GDP-[3H]mannose (Fig. 5). The in vitro products conmigrated with in vivo [3H]palmitate-labeled glycolipids A-like 1 and A-like 2 and, due to the lesser complexity of the in vitro as compared with the pattern of in vivo incorporations, the candidate bands corresponding to mannosylated intermediates could be identified as species I, III, and VI (Fig. 5, lanes 6–8). Pulse and chase in the in vitro system showed that the radioactivity gradually and proportionally accumulated on glycolipids A-like 1 and A-like 2 and, suggesting that they are different end products of the same route. Glycolipids A-like 1 and A-like 2 were always present in vivo and in vitro incorporations, although A-like 2 compared with A-like 1 appeared to be synthesized in a higher proportion in vivo than in vitro (compare Fig. 4, lanes 4 and 7, and Fig. 5, lanes 1–6).

It was also possible to characterize a component with very...
similar properties to glycolipid C of T. brucei, thus called glycolipid C-like. Among the in vitro synthesized species this was referred to as species VII and was the only one resistant to B. thuringiensis PI-specific PLC and sensitive to GPIPLD (Fig. 5, lanes 7 and 8). Following mild base treatment, species VII yielded a product, which co-migrated with lyso-glycolipid A-like 1 (Fig. 7, lanes 2 and 4), suggesting the loss of two acylations, one in position 2 of the sn-1-alkyl-2-acylglycerol and the other on the inositol ring (56). We have previously described a glycolipid C-like species using in vivo labeling with $^{3}$H-fatty acid, but despite its GPIPLD sensitivity and PLC resistance, it seemed atypically resistant to nitrous acid deamination (54). Taken together all of our data, it is reasonable to hypothesize that authentic nitrous acid-sensitive glycolipid C-like, which corresponds to in vitro glycolipid VII, is a minor component comigrating with multiple non-GPI species labeled in vivo or, alternatively, that a modified glucosamine could determine the refractory behavior of C-like in vivo to deamination.

Despite our previous reports of PLC resistance in 1G7-Ag (3, 26), structural investigations have unequivocally shown that such behavior was not due to acylation on the inositol ring (11). On the other hand, this modification seems to be a relevant feature for a subpopulation of Tc-BS glycoprotein of tissue-cultured trypomastigotes that is released in vesicles (64, 65). Preliminary experiments using a tissue-cultured trypanosome cell-free system showed species co-migrating with glycolipids C-like and A-like.3

The feasibility of synthesizing T. cruzi GPI in the cell-free system is a major technical advantage not only for better characterizing the biosynthetic steps but also for evaluating inhibitors affecting this pathway and, hopefully in the future, to test compounds that could stand as novel chemotherapeutic drugs. Since PMSF is known to block the addition of ethanolamine phosphate as well as inositol acylation in T. brucei (30, 31, 66), it was also tested in the T. cruzi in vitro system. In the presence of the inhibitor, the formation of A-like 1, A-like 2, and C-like was drastically prevented (Fig. 6), and despite the accumulation of intermediates in T. cruzi being less obvious than in T. brucei in the presented figure, experiments using shorter periods of pulse and chase have shown that radioactivity accumulates in species III and VI (see Fig. 5 for reference), which may correspond to Man$_3$GlcN-PI and Man$_2$GlcN-PI (Ref. 60).3 Therefore, ethanolamine transferases of African and South American trypanosomes might share some similarity regarding the catalytic site, and, given that the respective mammalian counterpart is not affected by this inhibitor (31), such enzymes definitely represent promising targets for chemotherapy.

As the GPI of 1G7-Ag is known to contain a minor proportion of PI-ceramide (11) and this component is present in 70% of the GPI of mucin-like proteins of metacyclic trypomastigotes (12), initially the difference in HPTLC migration between A-like 1 and A-like 2 was thought to possibly arise from one species bearing an sn-1-alkyl-2-acylglycerol and the other a PI-ceramide. Such a hypothesis was discarded based on the observation that base treatment affected equally the two glycolipids and yielded corresponding lyso-species still with the differential migration (Fig. 7). Moreover, the striking ubiquitous conservation of 1-O-hexadecyl-glycerol in glycoprotein anchors (6, 7, 10, 11, 27, 58), in glycoinositol phospholipids (8), and inositol phospholipids (59) of T. cruzi argues against a heterogeneity of the alkyl chain.

On the other hand, since glycolipid A-like 1 labels readily with $^{3}$H-ethanolamine whereas A-like 2 does not, the distinguishing feature between A-like 1 and A-like 2 could reside on the substitution on the third mannose distal from glucosamine. Considering, first, that part of the GPI population of epimastigote sialoglycoproteins contain an aminoethylphosphonic acid (AEP) replacing ethanolamine phosphate (EtNH$_2$PO$_4$) (10) and, second, that AEP biosynthesis is supposedly rather distinct from that of EtNH$_2$PO$_4$ (67, 68), it is quite reasonable to suggest, as depicted in Fig. 8, that glycolipid A-like 2 bears an AEP instead of an EtNH$_2$PO$_4$. Unfortunately the methodology so far employed to study the 1G7-Ag GPI was not powerful enough to clarify a putative heterogeneity in this position (6); therefore, it shall be useful in the future to refine these studies.

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3 N. Heise and M. L. Cardoso de Almeida, unpublished results.
employing a combination of $^{32}$P NMR and fast atom bombardment mass spectroscopy as described by Previato et al. (10). As observed before for the GPI anchor of the 1G7-Ag (11), the glycolipids A-like, as well as glycolipid C-like, are not labeled in the alkyl position of the glycerol (Fig. 3, lanes 7 and 14, and Table 1). Although such a finding could be indicative of a lipid exchange as described in T. brucei (69), we did not obtain experimental evidence for that in T. cruzi; therefore, efficient sn-1 position labeling might only be attainable when alkylglycerolipid biosynthesis in trypansomatids becomes better understood.

Taking into account that LPPG is extracted and co-migrates with glycolipid A-like in the solvent systems employed under our conditions (Table 1) and that there are around 1.5 $\times$ 10$^7$ copies of LPPG/epimastigote (5) and 1 $\times$ 10$^5$ LPPG-like molecule/MTC (29), it was puzzling to only find alkylacylglycerol in the alkyl position of the glycerol (Fig. 3, lane 14). Contreras, V. T., Salles, J. M., Thomas, N., Morel, C. M., and Goldenberg, S. (1988) Mol. Biochem. Parasitol. 30, 195–212. Ruiz, R. C., Rigone, V. L., Gonzales, J., and Yoshida, N. (1993) Infect. Immun. 61, 2041–2045.

Two basic biosynthetic routes are suggested, one starting with phosphatidylinositol and leading to glycolipids A-like 1 and A-like 2 tagged to anchor proteins and another departing from ceramide-PI and leading to LPPG. One of the hypothetical links between the two pathways is proposed at the level of Man$_2$GlcN$\alpha$-PI-glucosamine, and both might be transferred to proteins and possibly suffer further remodeling to ceramide. What advantage the different lipid moieties or special features such as AEP confer to the parasite remains open to conjecture.

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