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A glycosylphosphatidylinositol protein anchor from procyclic stage *Trypanosoma brucei*: lipid structure and biosynthesis

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Cells of the insect (procyclic) stage of the life cycle of the African trypanosome, *Trypanosoma brucei*, express an abundant stage-specific glycosylated phosphatidylinositol (GPI) anchored glycoprotein, the procyclic acidic repetitive protein (PARP). The anchor is insensitive to the action of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), suggesting that it contains an acyl-inositol. We have recently described the structure of a PI-PLC resistant glycosylphosphatidylinositol, PP1, which is specific to the procyclic stage, and have presented preliminary evidence that the phosphatidylinositol portion of the protein-linked GPI on PARP has a similar structure. In this paper we show, by metabolic labelling with [^{3}H]fatty acids, that the PARP anchor contains palmitate esterified to inositol, and stearate at *sn*-1, in a monoacylglycerol moiety, a structure identical to PP1. Using pulse–chase labelling, we show that both fatty acids are incorporated into the GPI anchor from a large pool of metabolic precursors, rather than directly from acyl-CoA. We also demonstrate that the addition of the GPI anchor moiety to PARP is dependent on *de novo* protein synthesis, excluding the possibility that incorporation of fatty acids into PARP can occur by a remodelling of pre-existing GPI anchors. Finally we show that the phosphatidylinositol (PI) species that are utilized for GPI biosynthesis are a subpopulation of the cellular PI molecular species. We propose that these observations may be of general validity since several other eukaryotic membrane proteins (e.g human erythrocyte acetylcholine esterase and decay accelerating factor) have been reported to contain palmitoylated inositol residues.

Key words: GPI anchor/lipid biosynthesis/PARP/procyclin/trypanosome

Introduction

Many membrane proteins from a wide variety of eukaryotic organisms are known to be linked to lipid bilayers by the covalent attachment of a glycosylated phosphatidylinositol (GPI) to the carboxyl terminus of the protein via an ethanolamine (EthN)–phosphate bridge (Ferguson and Williams, 1988; Cross, 1990; Thomas *et al.*, 1990). Protein-linked GPIs contain a conserved ethanolaminyl-phosphoinositolglycan, EthN-P-6Manα1–2Manα1–6Manα1–4GlcNα1–6-inositol-P, linking the C-terminal amino acid to an inositol phospholipid. The susceptibility of this structure to bacterial PI-specific phospholipase C (PI-PLC) is commonly used as a diagnostic for the presence of a GPI anchor (Ferguson and Williams, 1988). However, a number of proteins [e.g. human erythrocyte acetylcholine esterase (E^{hu}AChE, Roberts *et al.*, 1988), human erythrocyte decay accelerating factor (DAF, Walter *et al.*, 1990), procyclic acidic repetitive protein (PARP, Clayton and Mowatt, 1989), *Dictyostelium discoidium* antigen 117 (Sadeghi *et al.*, 1988) and contact site A glycoprotein (Stadler *et al.*, 1989)] and GPI lipids [e.g. P3, glycolipid C (Mayor *et al.*, 1990b; Krakow *et al.*, 1989) and PP1 (Field *et al.*, 1991)] contain the GPI moiety and are resistant to PI-PLC, probably because of a fatty acid esterified to the inositol (Roberts *et al.*, 1988).

Based on the identification of a series of incompletely glycosylated lipid species in radiolabelling experiments with bloodstream form trypanosome membrane preparations, it has been proposed that GPIs are constructed by sequential glycosylation of phosphatidylinositol (PI), followed by addition of ethanolamine phosphate (Masterson *et al.*, 1989; Menon *et al.*, 1990a, reviewed by Doering *et al.*, 1990; Field and Menon, 1991). The three mannose residues are derived from dolichol phosphorylmannose (Dol-P-Man) (Menon *et al.*, 1990b) and the glucosamine from UDP-GlcNAc (Doering *et al.*, 1989). In the final stage of assembly, both glycerol-linked fatty acids are remodelled to myristate to form the mature GPI structures P2 and P3 (Masterson *et al.*, 1989, 1990). P2 and P3 are identical except that P3 contains a palmitate esterified to the inositol (Mayor *et al.*, 1990b).

Studies of GPI biosynthesis in other eukaryotes suggest that acyl-inositol species may be more prevalent than initially thought. For example, in T cell hybridomas, an ethanolamine-containing intermediate has been characterized as PI-PLC resistant (De Gasperi *et al.*, 1990), whilst a species with the properties of an acyl-glucosamine PI has been identified in both a T cell hybridoma and a dolichol phosphomannose synthase deficient yeast mutant (Orlean, 1990; Sugiyama *et al.*, 1991). The presence of these GPI species raises the possibility that acylation occurs at an early stage in the glycosylation of PI, with deacylation being a late maturation step. However, these observations should be interpreted with caution as they have been made with mutant cells or cell free systems, and as such may not faithfully reflect the true physiological situation.

Selection of a particular PI for subsequent glycosylation and addition to protein may be complex. It has recently been reported that the molecular species of PI present in the GPI moiety of acetylcholinesterase from the electroplax organ of *Torpedo marmorata* are a minor subpopulation of the overall cellular PI molecular species (Butikofer *et al.*, 1990). Whilst it is probable that this reflects selectivity in the addition of monosaccharides to specific PI species, these authors noted that remodelling of the PI fatty acids after glycosylation, as seen in bloodstream form trypanosome GPIs, could not be ruled out as an explanation for their observations.

In the procyclic (insect midgut form) trypanosome the major cell surface protein, procyclic acidic repetitive protein (PARP) (Roditi *et al.*, 1989), has the properties of a PI-PLC resistant GPI anchored protein (Clayton and Mowatt, 1989). We have recently defined the structure of a major GPI, PP1, which is found in procyclic trypanosomes. This glycolipid contains a headgroup glycan of apparently identical structure to P2 and P3, but differs in the arrangement and composition of fatty acids. In contrast to P2 and P3, PP1 contains an *sn*-1, monostearylglycerol, rather than dimyristoylglycerol (Field *et al.*, 1991). Similarly to P3, PP1 has a palmitate esterified to the inositol (Mayor *et al.*, 1990b; Field *et al.*, 1991). It is important to note that the the actual palmitoylation position(s) on the inositol has not been defined to date. Evidence that PP1 is the precursor to the PARP GPI anchor has been obtained from two sources. Firstly, PP1 has been added to VSG in an *in vitro* system prepared from bloodstream trypanosomes (Mayor *et al.*, 1991) demonstrating that it is a substrate for the transfer machinery and, secondly, treatment of procyclics with millimolar mannosamine results in a coordinate shut-down of PP1 biosynthesis and addition of a GPI anchor to PARP, suggesting that biosynthesis of PP1 and the PARP anchor are parts of the same process (Lisanti *et al.*, 1991).

In this report we provide data on three further aspects of GPI anchor biosynthesis in procyclic trypanosomes. In the study of PP1 we obtained preliminary data on the lipid structure of the GPI anchors in procyclic trypanosomes. Based on co-chromatography on TLC, it appeared that the protein-linked GPI anchors had a similar lipid structure to PP1 (Field *et al.*, 1991). We have now examined this in detail and report that the PARP GPI anchor contains a PI moiety of apparently identical structure to PP1. We also investigated the process of inositol acylation, using pulse–chase experiments, and show that the palmitate esterified to the inositol is derived from a large pool of precursors, indicating that a direct acylation from acyl-CoA does not take place. Finally we show that a subpopulation of PI is utilized for GPI anchor biosynthesis.

Results

Structure of the phospholipid portion of the GPI anchor of PARP

Metabolic labelling of procyclic trypanosomes with [^{3}H]myristic acid and analysis by SDS–PAGE showed a single prominent band corresponding to PARP (Figure 1). A number of other trypanosome proteins were also labelled, at a minor level. Whilst some of these proteins may be GPI anchored, procyclic trypanosomes also attach fatty acids to proteins in other linkages (Schneider *et al.*, 1988). Analysis of the crude delipidated procyclic protein indicated that a considerable proportion of the radiolabel (>70%) was in fact due to labelling of GPI anchored proteins as indicated by sensitivity to nitrous acid and glycosylphosphatidyl-inositol-specific (GPI)-PLD treatment (Table I). The PI-PLC insensitivity of the labelled proteins is consistent with our previous observations that procyclic trypanosomes do not synthesize PI-PLC sensitive GPI lipids (Field *et al.*, 1991), and suggests that all the GPI anchored proteins in procyclic trypanosomes contain acyl-inositol. The near quantitative release of the radiolabel by base treatment demonstrated that the vast majority of the [^{3}H]fatty acids are present in ester linkage.

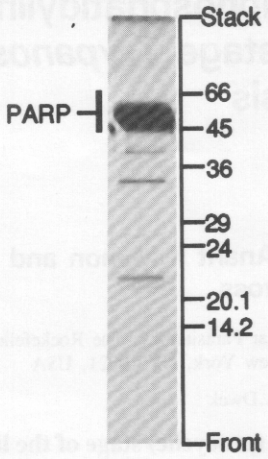

Fig. 1. PARP is the major protein labelled with [^{3}H]myristate in procyclic trypanosomes. Autoradiogram of a 15% SDS–polyacrylamide gel showing the incorporation of [^{3}H]myristic acid into total protein in procyclic *T.brucei*. Positions of molecular weight markers are indicated in kDa. The major band at ~50 kDa was assigned as PARP by co-migration with a purified [^{3}H]ethanolamine-labelled PARP sample (indicated by the bar).

Table I. Sensitivity of [^{3}H]myristic acid labelled proteins and purified PARP to GPI anchor cleavage reactions

Treatment	Percent cleaved		
	Total protein[a] Experiment number		PARP Experiment
	1	2	No. 1
Nitrous acid	74.6	78.9[b]	72.2
Control (buffer)	9.7	13.6	2.5
B.thuringiensis PI-PLC	18.3	14.6	4.0[c]
GPI-PLD (Serum)[d]	35.4	37.4	42.1
Control (buffer)	20.6	20.9	3.7
Base (NH_3/MeOH)	83.3	ND	90.5
Control (MeOH)	2.8	ND	ND

Total protein was delipidated by extensive extraction with chloroform/methanol/water (see Materials and methods), and resolubilized in 10% SDS. Purified PARP was solubilized in 2% CHAPS. Reactions were carried out using standard conditions (Mayor *et al.*, 1990a), and the reaction products were partitioned between water and butanol. Aliquots of each phase were assayed by liquid scintillation counting using ReadySafe™. In these experiments, radioactivity was initially contained in the aqueous phase, due to the covalent linkage of the PARP polypeptide to the radiolabelled GPI anchor moiety. Cleavage was indicated by recovery of radioactivity in the organic phase.

[a]Most (>80%) of the radiolabelled protein was PARP (see Figure 1)

[b]Two additions of sodium nitrite were made, at the beginning and after 16 h of incubation. Total reaction time was 20 h.

[c]PI-PLC released 99.2% of [^{3}H]inositol label from metabolically labelled rat liver PI.

[d]Note that the GPI-PLD is expected to release only the fatty acid attached to the glyceride moiety, and not that attached to the inositol.

Purification of [^{3}H]myristate-labelled PARP (Clayton and Mowatt, 1989, see Materials and methods) to radiochemical purity (data not shown) and re-analysis of the sensitivity of the fatty acid to release by various treatments (Table I) confirmed the presence of an acyl-inositol GPI anchor in PARP. The sensitivity of the incorporated radiolabel to base

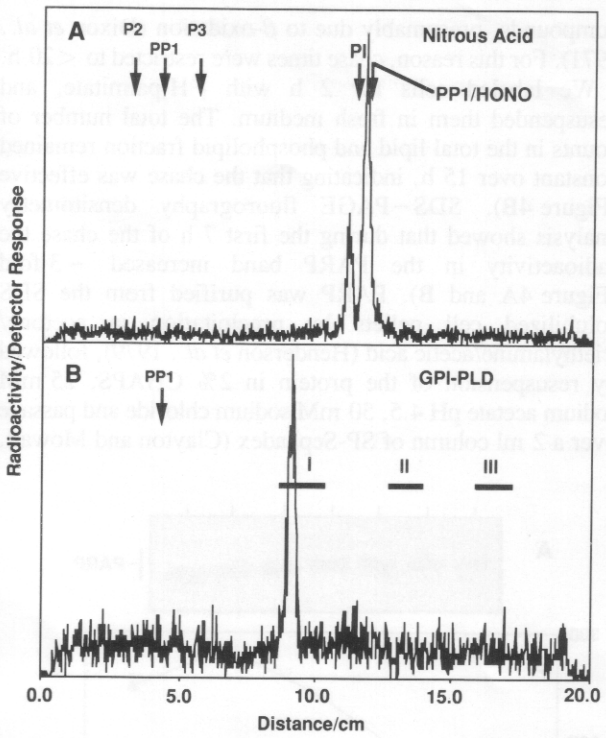

Fig. 2. The PARP GPI anchor contains lyso-PI, acylated on the inositol. **Panel A**: Thin layer chromatogram (system 1) showing the butanol-soluble products resulting from nitrous acid treatment of purified [^{3}H]myristate-labelled PARP. The major products migrate close to both a diacyl-PI standard and to the PI fragment derived by nitrous acid treatment of PP1 (PP1/HONO). **Panel B**; Thin layer chromatogram (system 1) of the butanol-soluble product following treatment of PARP with GPI-PLD (rabbit serum). A single product is released which co-chromatographs with a monoacyl-PA standard. Arrows: P2, P3, PI, and PP1 denote the migration positions of phosphatidylinositol-containing standard lipids. Bars: Migration positions of lyso-PA, PA and free fatty acid are indicated by I, II and III respectively. Origin and front at 2 and 18 cm respectively.

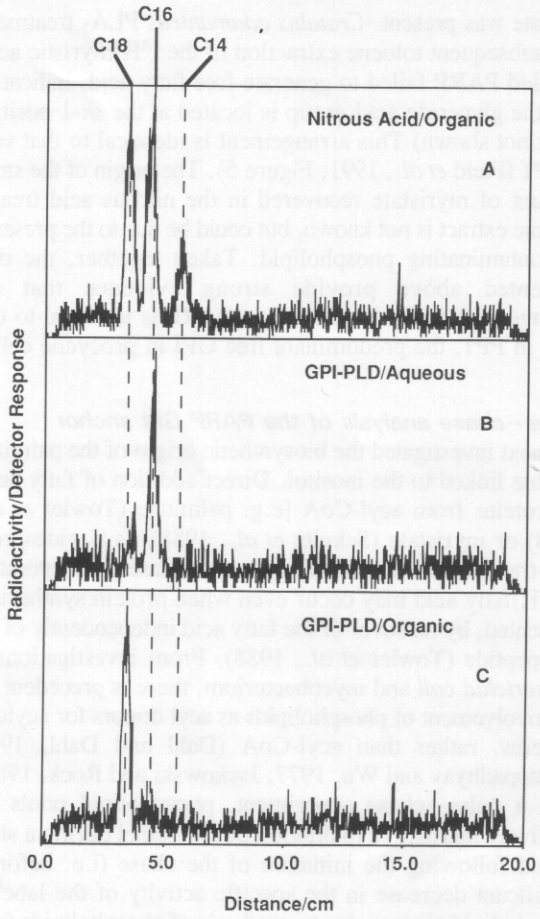

Fig. 3. The PARP GPI anchor contains stearate esterified to glycerol and palmitate esterified to inositol. Reversed phase high performance thin layer chromatograms of the fatty acid methyl esters released from fragments of the PARP GPI anchor. **Panel A**: Nitrous acid released fragment, containing the whole PI moiety. **Panel B**: Aqueous soluble portion following GPI-PLD cleavage, containing the inositol-linked fatty acid. **Panel C**: Butanol soluble portion following GPI-PLD cleavage, containing the glyceride. Origin and front at 2 and 18 cm respectively.

and nitrous acid hydrolysis indicated the presence of ester linked fatty acids and a non-acetylated glucosamine (Ferguson *et al.*, 1988), whilst resistance to PI-PLC and partial release of radiolabel by GPI-PLD was consistent with the assignment of the anchor as an acyl-inositol type GPI (Roberts *et al.*, 1988; Clayton and Mowatt, 1989; Mayor *et al.*, 1990b). We have incorporated [^{3}H]mannose, glucosamine and ethanolamine into PARP, in the presence of tunicamycin, suggesting that the anchor glycan contains the consensus components of GPI anchors (M.C.F. and A.K.M., unpublished observations) (Thomas *et al.*, 1990). The structure of the glycan portion of the PARP GPI anchor is currently the subject of detailed analysis and is not discussed further in this report.

Nitrous acid treatment of total [^{3}H]myristate-labelled proteins from procyclic trypanosomes releases a fragment which co-chromatographs with the analogous fragment obtained from PP1 (Field *et al.*, 1991). Analysis of [^{3}H]myristate-labelled PARP demonstrated that the radiolabel in the purified PARP was also contained in a moiety that co-chromatographed (TLC system 1) with the lipid fragment obtained from PP1 following nitrous acid treatment (Figure 2A, PP1/HONO). In this experiment we also obtained a second minor product, which was slightly more polar than the major one. This may be due to some complex aspect of the deamination reaction (see Mayor *et al.*, 1990b), and was not investigated further. Treatment of [^{3}H]myristic acid-labelled PARP with GPI-PLD, and TLC analysis of the released radioactivity demonstrated that the phosphatidic acid (PA) moiety was a mono-acylglycerol, based on co-chromatogaphy with authentic standards (Figure 2B).

The fatty acids present in [^{3}H]myristic acid-labelled PARP and in the GPI-PLD- and nitrous acid-cleaved fragments were identified by base hydrolysis, methylation and reversed phase TLC (see Materials and methods and Figure 3). Both stearate and palmitate are recovered from the PI moiety released by nitrous acid (Figure 3A). An identical profile was seen with the untreated PARP (not shown). From these data it is clear that the procyclic trypanosome can efficiently elongate exogenously added fatty acids (Dixon *et al.*, 1971), resulting in the recovery of [^{3}H]palmitate and [^{3}H]stearate from PARP derived from a [^{3}H]myristate labelling experiment. Palmitate was recovered from the aqueous phase following GPI-PLD treatment and butanol/water partitioning, indicating that this fatty acid remained linked to the protein after the action of the enzyme (Figure 3B), whilst stearate was recovered from the butanol phase, indicating that it was associated with the glyceride (Figure 3C). Because a lyso-PA was recovered from the GPI-PLD cleavage (Figure 2B), only a single

stearate was present. *Crotalus adamanteus* PLA_2 treatment, and subsequent toluene extraction of the [^{3}H]myristic acid-labelled PARP failed to generate free fatty acid, indicating that the glyceride acyl group is located at the *sn*-1 position (data not shown) This arrangement is identical to that seen in PP1 (Field *et al.*, 1991, Figure 5). The origin of the small amount of myristate recovered in the nitrous acid treated organic extract is not known, but could be due to the presence of contaminating phospholipid. Taken together, the data presented above provide strong evidence that the arrangement of the fatty acids in PARP is identical to that seen in PP1, the predominant free GPI in procyclic cells.

Pulse–chase analysis of the PARP GPI anchor

We next investigated the biosynthetic origin of the palmitate residue linked to the inositol. Direct addition of fatty acids to proteins from acyl-CoA [e.g. palmitate (Towler *et al.*, 1988) or myristate (Schultz *et al.*, 1988)] is a widespread post-translational modification. In these cases, incorporation of [^{3}H]fatty acid may occur even when protein synthesis is prevented, by turnover of the fatty acid independently of the polypeptide (Towler *et al.*, 1988). From investigations in *Escherichia coli* and mycobacterium, there is precedent for the involvement of phospholipids as acyl donors for acylated proteins, rather than acyl-CoA (Dahl and Dahl, 1984; Chattopadhyay and Wu, 1977; Jackowski and Rock, 1986).

In a pulse–chase experiment, phospholipid pools are relatively insensitive to dilution of radiolabel during a short period following the initiation of the chase (i.e. before a significant decrease in the specific activity of the labelled phospholipids occurs due to synthesis of phospholipids from cold precursors or degradation of the labelled lipids). If a phospholipid is acting as an acyl donor, detectable radiolabelled acyl groups will continue to be transferred following dilution of the pulse radiolabel. In contrast, fatty acyl-CoA pools will be rapidly diluted by the addition of excess cold fatty acid, so that incorporation from acyl-CoA will stop rapidly after the initiation of the chase (Dahl and Dahl, 1984). Therefore a stable acyl-group acceptor, e.g. a GPI anchored protein, will be seen to continue to incorporate radiolabel if that label is provided from a phospholipid source, but will cease incorporation if the label is derived from fatty acyl-CoA.

PARP is a suitably stable protein as the half-life is >24 h (Clayton, 1988; P.Patnaik, personal communication) and we therefore expected that pulse–chase labelling of PARP with [^{3}H]palmitate would be informative. Regardless of the origin of the inositol-linked palmitate, it was expected that incorporation of radioactivity into PARP would increase during the chase period due to continued synthesis of GPI, derived from a [^{3}H]stearate containing pool of PI. Therefore we determined the relative amount of both palmitate and stearate in the PARP GPI anchor in order to ascertain the labelling kinetics of both fatty acids.

We first analysed the kinetics of incorporation of [^{3}H]palmitate into phospholipids and protein. As expected, incorporation increased with time in both protein and lipid, with a PARP band becoming easily detectable after 2 h (not shown). Analysis of the [^{3}H]fatty acids now present in lipid and protein showed that the [^{3}H]palmitate was converted to [^{3}H]stearate in a time-dependent manner (not shown). Over longer periods of time (>24 h), we observed that the ^{3}H label became increasingly converted into more polar compounds, presumably due to β-oxidation (Dixon *et al.*, 1971). For this reason, chase times were restricted to <20 h.

We labeled cells for 2 h with [^{3}H]palmitate, and resuspended them in fresh medium. The total number of counts in the total lipid and phospholipid fraction remained constant over 15 h, indicating that the chase was effective (Figure 4B). SDS–PAGE fluorography densitometry analysis showed that during the first 7 h of the chase the radioactivity in the PARP band increased ~3-fold (Figure 4A and B). PARP was purified from the SDS solubilized cell pellets by precipitation in acetone/triethylamine/acetic acid (Henderson *et al.*, 1979), followed by resuspension of the protein in 2% CHAPS, 25 mM sodium acetate pH 4.5, 50 mM sodium chloride and passage over a 2 ml column of SP-Sephadex (Clayton and Mowatt,

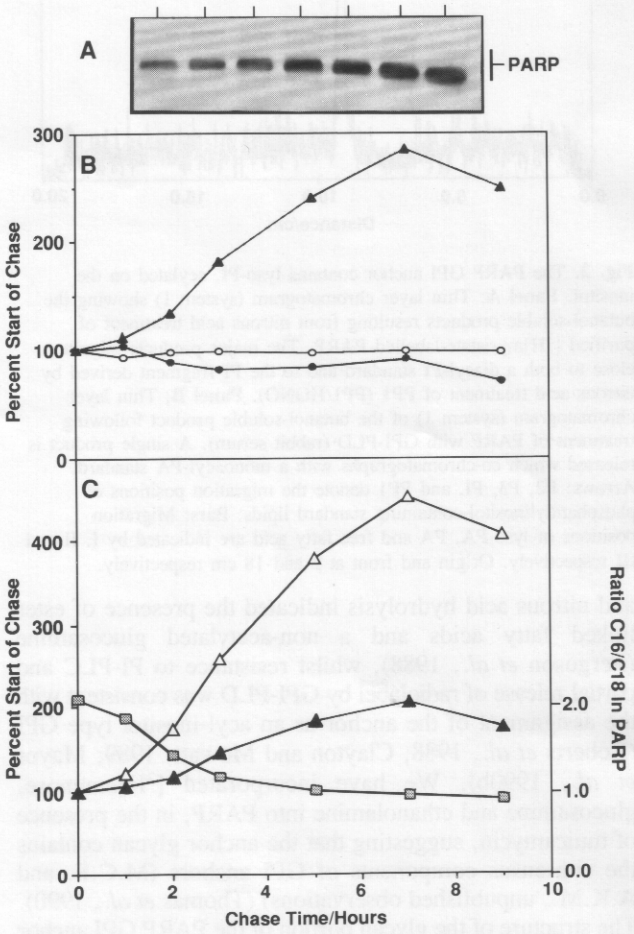

Fig. 4. Pulse–chase labelling with [^{3}H]palmitate; continuous incorporation of fatty acids into PARP in the chase period. **Panel A**: PARP region of fluorogram of 15% SDS–polyacrylamide gel of pulse–chase experiment showing continuous incorporation of radiolabel into PARP during chase. **Panel B**: Radioactivity associated with PARP, total lipid and phospholipid fraction during chase period after a 2 h pulse with [^{3}H]palmitate. PARP data (solid triangles) from densitometry of SDS gel shown in panel A; total lipid (open circles) from radioactivity associated with a chloroform/methanol/water extraction of procyclic cells; phospholipid (closed circles) from TLC (system 3). **Panel C**: Amount of [^{3}H]palmitate (closed triangles) and stearate (open triangles) in PARP during chase. Data were obtained from the ratio (shaded squares) of the two fatty acids recovered by FAMEs analysis and the total amount of radioactivity in PARP. Note that both stearate and palmitate increase during the chase. Further experimental details and numerical data are presented in Table II (experiment number III).

1989) equilibrated in the same buffer. Analysis of an aliquot of the material by SDS−PAGE fluorography showed that the PARP was radiochemically pure following the single column step (data not shown). The [^{3}H]fatty acid composition of PARP at the different time points of the chase was then assessed (see Materials and methods). Similarly to the situation seen in continuous labelling, the ratio of palmitate to stearate decreased during the chase, equilibrating after 5 h at ~1:1 palmitate to stearate, reflecting the elongation of the input radiolabel (Figure 4C). However, the increase in total radioactivity in PARP, as measured by densitometry, outweighed the change in distribution of the radiolabel (Figure 4C), so that the radiolabel in both palmitate and stearate clearly increased during the chase (Figure 4C). We observed that the stearate label in PARP increased almost 5-fold after 7 h, compared with 2-fold for the palmitate. Data from this and two other similar experiments are shown in Table II.

Table II. Incorporation of ^{3}H into PARP during chase

	Time (h)						
Experiment I	0.0		3.3		13.5		
PARP	100.0#		251.2		1184.5		
Palmitate[a]	100.0		196.3		788.9		
Stearate	100.0		350.0		1896.7		
	Time (h)						
Experiment II	0.0	1.0	3.3	4.8	19.3		
PARP	100.0	112.3	244.3	187.0	260.4		
(+ cycloheximide)	100.0	116.0	94.2	80.2	68.7		
	Time (h)						
Experiment III[b]	0.0	1.0	2.0	3.0	5.0	7.0	9.0
PARP	100.0	111.3	134.2	181.7	240.5	285.0	249.4

Data from three experiments are summarized. All experiments were conducted as described in Materials and methods. Relative radioactivity in PARP was determined by densitometry of fluorograms from SDS gels. Data are the mean of two gels for each point (to minimize aretefacts due to differential loading), except experiment I, where a single gel was analysed. Experiments I and II; 4 h pulse, Experiment III; 2 h pulse. Fatty acid composition of PARP was analysed for expriments I (this table) and III (see Figure 3).

Data are expressed as the optical density of the PARP band on a fluorogram of an SDS−PAGE gel, normalized to time zero as 100%.

[a] Values for radioactivity recovered from metabolically labelled PARP as palmitate and stearate are given (see Materials and methods).

[b] Data for experiment III are presented graphically in Figure 3.

Attempts to inhibit the conversion of palmitate to stearate by treating the trypanosomes with cerulenin, a compound shown to inhibit fatty acid synthase in a wide variety of organisms (Omura, 1986; Vance *et al.*, 1972), were unsuccessful. Cell viability was compromised before an effective inhibition of fatty acid elongation was seen.

Analysis of the organic extract on TLC system 3 resolved the free fatty acid from the other labelled species. A constant and very low level (<0.1% of total lipid associated radioactivity) of free [^{3}H]fatty acid was seen during the chase. The culture medium contains free fatty acid in 100−1000 fold excess (~100 μM) over the initial radiolabel, so the contribution of re-utilized fatty acid to the labelling of PARP is negligible. These observations are in contrast to those made on biosynthesis of the bloodstream form trypanosome variant surface glycoprotein (VSG) where incorporation of [^{3}H]myristate is halted immediately following initiation of a chase (Krakow *et al.*, 1986). Because we have demonstrated that [^{3}H]stearate continues to be added to newly synthesized PARP, remodelling via fatty acyl-CoA is also excluded as a step in biosynthesis of the PARP GPI anchor. From the above experiments, we conclude that the palmitate residue esterified to inositol is not derived directly from palmitoyl-CoA, but from a large pool of precursors.

Table III. Analysis of distribution of [^{3}H]fatty acids After incorporation of [^{3}H]palmitate into phosphatidylinositol

Chase time (h)		C.p.m. recovered (%)		
		DAG[a]	*sn*-1[b]	*sn*-2
0	C_{16}[c]	71.4	72.6	59.1
	C_{18}	28.6	27.4	40.9
3	C_{16}	75.7	92.5	73.1
	C_{18}	24.3	7.5	26.9
9	C_{16}	90.5	91.6	77.7
	C_{18}	9.5	8.4	22.3

[a]Diacylglycerol (DAG) was generated from TLC purified PI by digestion with *B.thuringiensis* PI-PLC (see Materials and methods). Cleavage was >80% in all cases.

[b]The fatty acid at *sn*-2 was released from TLC purified PI by treatment with *C.adamanteus* PLA_2. The released fatty acid was recovered in the toluene phase of a water:toluene partitioning procedure. Lyso-PI was retained in the aqueous phase (determined from partitioning of identically treated [^{3}H]inositol-labelled rat microsome PI). Release of the *sn*-2 fatty acid was ~80% in all cases.

[c]Fatty acid assignments were made on the basis of co-chromatography with standard compounds. Co-migration of unsaturated fatty acids with saturated species cannot be formally ruled out. The methylester of arachidonate, a possible complex product from stearate, migrates with an R_f of ~0.4, well ahead of the two species detected here.

Addition of fatty acids to PARP requires de novo protein synthesis

Proteins that are directly acylated by the action of fatty acyl-CoA can incorporate fatty acids well after synthesis of the polypeptide has occurred (Towler *et al.*, 1988), by turnover of the fatty acyl moiety independently of the polypeptide. In the case of a GPI anchored protein, fatty acid could be incorporated during the chase by remodelling of the fatty acids or of a more extensive portion of the anchor. To exclude these possible explanations for the continued incorporation of [^{3}H]fatty acid into PARP during the chase, we performed the pulse−chase experiment in the presence of the protein synthesis inhibitor cycloheximide. Cells were labelled for 4 h with [^{3}H]palmitate and the radiolabel was then washed out and the culture split into two aliquots (see Materials and methods). One culture was incubated as a control, the other was treated with cycloheximide.

We observed that during the pulse, radioactivity in PARP increased steadily, and this continued in the control culture during the chase (Table II). Inclusion of cycloheximide in the chase medium prevented the continued radiolabelling of PARP, demonstrating that incorporation of radiolabelled fatty acid in the chase was dependent on *de novo* protein

synthesis (Table II). This observation is consistent with the addition of the GPI anchor being a rapid post-translational event, and excludes the possibility that extensive fatty acid remodelling occurs after the GPI anchor is added to PARP. This is similar to the effect on [^{3}H]myristate incorporation into VSG in bloodstream trypanosomes treated with cycloheximide (Ferguson *et al.*, 1986).

Radiolabelled fatty acid composition of PI

Analysis of the fatty acids present in the total lipid fraction obtained with chloroform/methanol demonstrated that the input radiolabel was efficiently converted to stearate and myristate in a time-dependent manner (data not shown). We analysed the fate of [^{3}H]palmitate incorporated into purified PI (see Materials and methods) after 0, 3 and 9 h of chase. These data are presented in Table III. [^{3}H]palmitate was the predominant fatty acid recovered from all the PI samples analysed. Separation of the fatty acids at *sn*-1 and *sn*-2 by PLA_2 treatment also demonstrated that palmitate was predominant, with small amounts of stearate recovered in all cases. Because our pulse–chase data suggest that remodelling of the PI does not occur, these data suggest that the PI that is selected for glycosylation is a subpopulation of the metabolically labelled PI.

Discussion

In this report we show that the GPI anchor of PARP contains a novel PI structure, 1-stearylglycerol with palmitate in ester linkage to inositol (Figure 5). This structure is identical to that reported for the major GPI, PP1, identified in procyclic trypanosome extracts (Field *et al.*, 1991). Whilst definitive proof of a precursor–product relationship between PP1 and the PARP GPI anchor is not available, this observation, taken together with other recent studies on *in vitro* addition of PP1 to VSG (Mayor *et al.*, 1991), and the simultaneous inhibition of PP1 biosynthesis and addition of GPI to PARP by mannosamine (Lisanti *et al.* 1991), lends considerable weight to the hypothesis that this is indeed the case.

Analysis of the whole cell protein after labelling with [^{3}H]myristate identified only comparatively minor species in addition to PARP (Figure 1). Resistance of the radiolabelled fatty acids to PI-PLC treatment demonstrated that no PI-PLC sensitive GPI anchors are synthesized in the procyclic trypanosome, in agreement with the exclusive presence of acyl-inositol GPIs reported in procyclics (Field *et al.*, 1991).

The structure of the PARP GPI anchor is radically different to that observed for the GPI anchor of VSG from bloodstream form *T.brucei* (Ferguson *et al.*, 1988), where the PI is dimyristoyl. These observations confirm our previous speculations concerning differences in the biosynthesis of GPIs in procyclic and bloodstream stages of this parasite (Field *et al.*, 1991; Field and Menon, 1991), and provide the first description of a developmental alteration in protein-linked GPI structure. The structure of the glycan from the PARP GPI anchor is currently not known, but preliminary investigations suggest that it is extensively modified from the Man_3GlcN core glycan identified in PP1 (M.C.F., A.K.M. and M.A.J.Ferguson, unpublished observations), and may also be highly heterogeneous.

Pulse–chase experiments with [^{3}H]glucosamine (utilized as UDP-GlcNAc) indicate that PP1 is metabolically stable, with a half-life of >24 h (M.C.F., unpublished observations), and is unaffected by inhibition of protein synthesis. We suggest that these observations are due to escape of PP1 from the endoplasmic reticulum (ER) into the Golgi or plasma membrane, where the glycolipid can no longer be added to protein. We have observed the presence of larger GPI headgroups in procyclic trypanosomes (Field *et al.*, 1991), possibly generated by glycosylation of PP1 by Golgi glycosyltransferases. The presence of α-galactosylated GPIs in bloodstream form trypanosomes may be the consequence of a similar phenomenon (S.Mayor, A.K.M. and G.A.M.C. manuscript submitted). These observations are consistent with inefficient ER retention of GPIs, and processing in a post-ER compartment.

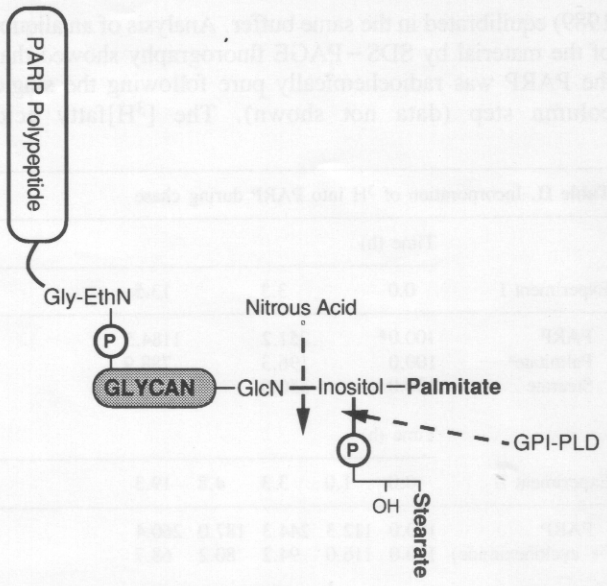

Fig. 5. Proposed partial structure of the PARP GPI anchor. The structure of the phosphatidylinositol portion, as determined in this report, is shown. The positions of action of nitrous acid and GPI-PLD are indicated by arrows. The glycan, represented by the shaded box, has not been defined at the structural level. The C-terminal amino acid of the PARP polypeptide, amide-linked to ethanolamine, has been proposed to be glycine (Clayton and Mowatt, 1989).

The origin of the palmitate residue on the inositol is of particular relevance, as this structural feature may be biosynthetically important (see below) and effectively divides GPI anchored proteins into two subclasses, the PI-PLC sensitive and resistant forms [it should be noted that the presence of palmitoylinositol has not been demonstrated in all PI-PLC resistant GPIs and, in the folate binding protein, fatty acid esterification of the GPI inositol does not appear to confer PI-PLC resistance (Luhrs and Slomiany, 1989)]. As the position of the inositol-linked palmitate has not been determined for any GPI (lipid or protein-linked), the precise correlation between PI-PLC resistance and inositol palmitoylation is not known.

Our pulse–chase studies are consistent with the view that the palmitate is derived from a large precursor pool, for example a phospholipid, and not directly from acyl-CoA. We have also demonstrated that the addition of fatty acids to PARP requires ongoing protein synthesis. The stage at which the inositol becomes palmitoylated is not known, but recent observations in other eukaryotes suggest that this step may occur early in the pathway (Orlean, 1990; DeGasperi *et al.*, 1990; Sugiyama *et al.*, 1991). It has been proposed that acylation of the inositol is an important step in altering the conformation of the PI headgroup and allowing selection

of a subpopulation of PI molecules for glycosylation. Our own observations with a procyclic trypanosome membrane preparation suggest that inositol acylation of the nascent GPI occurs after synthesis of the GlcN-PI in this organism (M.C.F. and A.K.M., unpublished observation). Alternatively, acylation of GlcN-PI could be a signal for the further elaboration of the glycolipid, and may be a synthesis control point.

Our data also suggest that a subpopulation of PI molecules is selected for glycosylation, as we observed that the majority of the [^{3}H]fatty acids at *sn*-1 in the metabolically-labelled PI were palmitate, with a small amount of stearate, whereas in our analysis of PP1 (Field *et al.*, 1991) and PARP we recovered exclusively stearate at this position. In fact, we observed that the proportion of stearate at *sn*-2 actually decreased during the chase, which may reflect the utilization of this population of PI molecules (the major route may be in intracellular signalling, rather than GPI biosynthesis). We have recently identified a diacylglycerol GPI lipid in *in vitro* experiments with procyclic trypanosomes with the properties of a PP1 precursor (M.C.F. and A.K.M., unpublished observations), consistent with a diacyl-PI being the ultimate GPI precursor. These observations are similar to the report that the molecular species of the PI in the GPI anchor of acetylcholinesterase of *Torpedo marmorata* are minor components of the cellular PI pool (Butikofer *et al.*, 1990).

The positive identification of the fatty acid donor is beyond the scope of the present paper and will require the development of an *in vitro* system able to utilize exogenous phospholipids efficiently as acyl donors. In this regard, it is interesting to note that, in *M.capricolum* and *E.coli*, a lack of specificity for the acyl donor has been noted (Dahl and Dahl, 1984; Lai and Wu, 1980) and, as a wide spectrum of [^{3}H]fatty acid-labelled phospholipids are synthesized by the procyclic trypanosome, several candidate acyl donors exist.

Preliminary attempts to label P3 and acyl PI-linked GPI intermediates via [^{3}H]palmitoyl-CoA in bloodstream form trypanosome membrane preparations were unsuccessful (Menon *et al.*, 1990a). These results were unexpected at the time since a closely analogous reaction, the acylation of mycobacterial PI-dimannoside (Brennan and Ballou, 1968), uses fatty acyl-CoA to generate a hyperacylated PI-dimannoside containing two extra fatty acids, one esterified to the inositol residue and another esterified to an inositol-linked mannose (Ballou, 1972). The observations made in this report are consistent with acylation of GPI inositol being the consequence of a transacylation between the GPI and a glycerophospholipid. Phospholipid fatty acid donors have been identified in a number of bacterial transacylation reactions including the amino-terminal N-acylation of the major outer membrane lipoprotein in *E.coli* (Jackowski and Rock, 1986), the acylation of a spectrum of membrane proteins in *M.capricolum* (Dahl and Dahl, 1984), and the synthesis of acyl phosphatidylglycerol (Cho *et al.*, 1977). It is also formally possible that GPI acylation involves an acyl-protein donor, analogous to the acyl-carrier proteins identified in *E.coli* (Raetz and Dowhan, 1990).

Materials and methods

Culture and metabolic labelling of trypanosomes

Cells of the culture adapted procyclic form *Trypanosoma brucei* strain 427, grown in SDS-79 medium, were used throughout this study (Brun and Schonenberger, 1979). Metabolic labelling was performed as previously described (Field *et al.*, 1991). Labelling with [^{3}H]palmitate (40–60 Ci/mmol, Amersham) was performed exactly as reported for [^{3}H]myristate labelling (Field *et al.*, 1991). Cycloheximide (Sigma) treatment was done at 33 μg/ml, a concentration determined to inhibit protein synthesis in these cells (as assayed by incorporation of [^{35}S]methionine into trichloroacetic acid precipitable counts) by 95% (M.C.F. and A.K.M., unpublished observations).

Analysis of the GPI anchors of procyclic proteins

Trypanosomes (5×10^9 cells) were metabolically labelled by overnight incubation with [^{3}H]myristate. Following labelling the cells were harvested and washed with ice-cold phosphate buffered saline (PBS). Lysis was performed by exhaustive organic extraction, first with ice-cold chloroform:methanol (2:1 v/v), followed by ice-cold chloroform:methanol:water (10:10:3 v/v/v) using the two step extraction procedure given below. The delipidated pellet (1×10^7 c.p.m.) was solubilized in 10% SDS, and an aliquot analysed by SDS–PAGE. An aliquot (5%) of this 'total' protein material was precipitated with acetone/triethylamine/acetic acid (Henderson *et al.*, 1979) and redissolved in 50 μl 0.4% NP-40 (Calbiochem) in water. The protein was solubilized by boiling and 50 μl Tris-buffered saline (TBS) was added. Aliquots of this material were analysed for the presence of [^{3}H]myristate-labelled GPI anchored proteins by enzymatic and chemical procedures (see below and Table I).

Analysis of the metabolically labelled GPI anchor of PARP

The remainder of the delipidated protein was lyophilized and precipitated with 1 ml ice-cold ethanol. Approximately 20% of the radioactivity in the initial protein pellet was recovered at this stage (the remainder of the radioactivity being incorporated into lipid species). The precipitate was washed twice with cold ethanol, five times with acetone/triethylamine/acetic acid (Henderson *et al.*, 1979) and finally once with acetone. The pellet was then extracted with 4% CHAPS, and the extract mixed with an equal volume of 50 mM sodium acetate pH 5.5, and applied to a column of SP-Sephadex (Pharmacia-LKB), equilibrated in 25 mM sodium acetate, 50 mM NaCl, 2% CHAPS (Clayton and Mowatt, 1989). All radioactivity was recovered in the flowthrough as no additional ^{3}H counts were eluted with a 1 M NaCl wash. The flowthrough was pooled and applied to a column of ConA Sephadex (Pharmacia-LKB). In this case, 25% of the counts were retained by the column and subsequently eluted with 200 mM α-methylmannoside (Sigma), the remainder being unbound by the lectin. Both the bound and unbound fractions were concentrated by acetone precipitation. SDS–PAGE fluorography analysis of the purified PARP (bound) showed only a single band at ~50 kDa, demonstrating that the additional labelled proteins had been removed. The unbound fraction from the lectin column did not contain PARP as the radioactivity migrated as a smear at or close to the front when run on an SDS gel. To the purified PARP, 5 mg of fatty acid free BSA (Boehringer-Mannheim) was added, and the mixture dialysed against 10% methanol/5% formic acid. The retentate was concentrated in a Speedvac evaporator (Savant Instruments) to 2 ml, and finally washed with water-saturated butanol to remove residual phospholipids and detergents. Aliquots (2000 c.p.m. per assay) were then analysed by chemical and enzymatic cleavage (Table I). The efficacy of these procedures was determined by butanol/water partitioning of the reaction products (Mayor *et al.*, 1990a). Release of radiolabel, by recovery of counts in the butanol phase, indicated that cleavage of the GPI anchor had taken place. To determine the positions of the fatty acids in the GPI anchor, aliquots (15 000 c.p.m.) were subjected to nitrous acid treatment (to release the entire PI moiety), or GPI-PLD cleavage (to release the glyceride moiety alone). Aliquots of the organic soluble products were analysed by TLC (system 1). The butanol and aqueous phases obtained following these reactions, as well as an untreated PARP sample, were then separately analysed as fatty acid methyl esters as described (Mayor *et al.*, 1990b), using TLC system 2.

Lipid extractions and protein solubilization

Lipids were extracted into two separate fractions as described (Menon *et al.*, 1990a). The first extract, obtained with chloroform/methanol 2:1 (v/v) (extract 1), contains the phospholipid species together with less polar GPI lipids, whilst the second extract, obtained with chloroform/methanol/water 10:10:3 (v/v/v) (extract 2) contains the more polar ethanolamine-containing GPI lipids. Folch washing of extract 1 and partitioning of extract 2 between butanol and water were performed as described (Menon *et al.*, 1990a).

Alternatively, lipids were extracted in a single step by the addition of 330 μl chloroform:methanol (1:1 v/v) to 50 μl aqueous cell pellet (final composition chloroform:methanol:water 10:10:3 v/v/v). The extract was removed following centrifugation and the pellet reextracted twice with 500 μl chloroform:methanol:water (10:10:3 v/v/v). Both extracts were combined, dried in a Speedvac evaporator, and partitioned between water and butanol. Lipids were recovered in the butanol phase.

Phosphatidylinositol was purified from the chloroform/methanol extracts of cells metabolically labelled in a pulse–chase experiment with [^{3}H]palmitic acid (see below). Extracts were initially chromatographed on TLC using system 1, and the peak which co-migrated with a PI standard was isolated. The purified lipid was analysed using two further TLC systems (systems 4 and 5), and in each case a single peak was observed which co-chromatographed with the PI standard. TLC system 5 will resolve PI and phosphatidylserine (PS), which co-chromatograph on TLC system 1, and therefore this analysis, together with the near complete sensitivity of this material to *Bacillus thuringiensis* PI-PLC (see Table III), demonstrated that the PI was radiochemically pure.

In experiments where the incorporation of radioactivity into protein was to be assayed, the delipidated pellet was taken up in 50–100 μl of 5% SDS, sonicated for 15 min, and incubated overnight at room temperature.

Pulse–chase experiments

Cells were incubated at 27°C during both the pulse and the chase. In experiments where fatty acid label was pulse–chased, cells were first incubated in fatty acid labelling medium (Field *et al.*, 1991) at 4×10^7 cells/ml and, after the pulse period, the cells were pelleted, washed twice with PBS or medium, and then resuspended in fresh SDM 79, 10% fetal calf serum (FCS, containing 10 μM non-esterified fatty acid [Tissue Culture Association Manual, **1**(4) (1975)], representing a 500-fold dilution of the radiolabel) and 7.5 μg/ml hemin at $1-2\times10^7$ cells/ml. For glucosamine labelling, an excess of cold glucosamine (1000-fold, 2 mM) was added to the culture to initiate the chase. At each time point, an aliquot was taken from the culture, cells were pelleted in a microfuge and lysed by addition of chloroform:methanol (see lipid extraction). Protein was solubilized in SDS, and samples were stored at 4°C.

Enzyme and chemical treatments.

Nitrous acid deamination, sodium hydroxide/methanol base hydrolysis, ammonia/methanol (mild) base hydrolysis, and enzyme digestions with GPI-phospholipase D (rabbit serum), *B.thuringiensis* PI-PLC (gift of Dr M.Low, Columbia University, New York) and *Crotalus adamanteus* phospholipase A_2 (Sigma) were all performed as described previously (Mayor *et al.*, 1990a,b). In some cases, the reaction volumes were increased so that the residual detergent concentration would be lowered. Where necessary, reaction products were partitioned between water and water-saturated *n*-butanol. The reaction mixture was acidified with 10 μl glacial acetic acid, and made up to a total volume of 0.5 ml with distilled water. The sample was then extracted twice with 0.5 ml water-saturated *n*-butanol, and the organic phases pooled.

For analysis of PI, samples were treated with the appropriate enzyme, and the products were partitioned between toluene/water. Diacylglycerol from PI-PLC cleavage, and free fatty acid were recovered in the toluene phase, whilst lyso-PI was retained in the aqueous phase.

Chromatography

Thin layer chromatography was performed in pre-equilibrated tanks, and plates were typically developed 16 cm from the origin under the following conditions; glass backed silica 60 plates (Merck), chloroform/methanol/water (10:10:2.5, v/v/v) (system 1), or petroleum ether/diethylether/acetic acid (70:30:2, v/v/v) (system 2), or on glass backed high performance reverse phase (C_{18}) plates (Merck), acetonitrile/acetic acid (1:1, v/v) (system 3). PI was analysed for purity using Si60 plates developed with chloroform/methanol/acetic acid/water (25:15:4:2, by vol) (system 4) or chloroform/acetone/methanol/acetic acid/water (45:16:16:11:6, by vol) (system 5). Radioactivity was visualized by scanning the plates with a Berthold Model LB2842 linear scanner. Fatty acid methyl ester standards were visualized by spraying the TLC plate with 2% α-cyclodextrin (Sigma) and exposing the plate to iodine vapour.

Miscellaneous methods

Liquid scintillation spectroscopy was performed with ReadySafe™ scintillation fluid (Beckman). SDS–PAGE in 15% gels was as described by Laemmli (1970). After electrophoresis, gels were stained with Coomassie blue and destained in 40% methanol, 10% acetic acid (v/v), and then impregnated with EN^{3}HANCE (Dupont) and dried for fluorography. TLC plates for fluorography were sprayed with EN^{3}HANCE reagent (Dupont). Fluorography was performed at −80°C with two intensifying screens and Kodak X-OMAT AR film. Lyso-PA standards were obtained by digestion of PA standards (Sigma) with *C.adamanteus* PLA_2. 1-stearoyl, 2-arachidonylglycerol and fatty acid methyl ester standards were purchased from Sigma. Standard PI and PS were produced by [^{3}H]inositol labelling of rat liver microsomes and [^{3}H]serine labelling of bloodstream form trypanosomes respectively.

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