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Phenylmethanesulphonyl fluoride inhibits GPI anchor biosynthesis in the African trypanosome

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A wide variety of eukaryotic membrane proteins are anchored to the outside of cells by covalent linkage to glycosyl phosphatidylinositol (GPI). One of the best characterized examples is the variant surface glycoprotein (VSG) of the protozoan parasite, *Trypanosoma brucei*. The structure of the GPI precursor is ethanolamine-PO_4-Manα1-2Manα1-6Manα1-4GlcNH_2-PI; the phosphoethanolamine moiety forms an amide linkage to the VSG polypeptide α-COOH group during its attachment to protein. Here we report that the serine esterase inhibitor, phenylmethanesulphonyl fluoride (PMSF), inhibits phosphoethanolamine incorporation into the GPI precursor resulting in the accumulation of a Man_3GlcNH_2-PI intermediate. PMSF exerts this effect both in living trypanosomes and in a trypanosome-derived cell-free system. This is the first report of an inhibitor which affects GPI biosynthesis but not *N*-glycosylation. A model of the mechanism of phosphoethanolamine incorporation into the GPI precursor, based on the known properties of PMSF, is presented.

Key words: GPI biosynthesis/membrane protein/phosphoethanolamine incorporation/PMSF/trypanosomes

Introduction

Organisms as diverse as protozoa and man use covalent linkage to glycosyl phosphatidylinositol (GPI) for anchoring proteins to cell surfaces (Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Thomas *et al.*, 1990). The GPI moiety has been implicated in a number of processes, including protein targeting in polarized cells (Lisanti *et al.*, 1989; Dotti *et al.*, 1991), T cell activation (Robinson *et al.*, 1989), and a novel form of endocytosis that is clathrin-independent (Rothberg *et al.*, 1990).

The basic structure of GPI anchors is conserved between different eukaryotes (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Roberts *et al.*, 1988; Schneider *et al.*, 1990), suggestive of a shared biosynthetic pathway (Doering *et al.*, 1990; Ferguson and Homans, 1989). The relative abundance of *Trypanosoma brucei* variant surface glycoprotein (VSG) has made the African trypanosome the system of choice for the study of GPI biosynthesis (Doering *et al.*, 1990). In the bloodstream form of *T. brucei* the anchor is constructed as a GPI precursor, the structure of which is ethanolamine-PO_4-Manα1-2Manα1-6Manα1-4GlcNH_2-PI (Mayor *et al.*, 1990b). This precursor is attached to newly-made VSG within a minute of the polypeptide being synthesized. The attachment requires the removal of a COOH-terminal GPI addition signal peptide and formation of an amide linkage between the phosphoethanolamine moiety of the GPI precursor and the newly exposed α-COOH group of the VSG polypeptide. Studies using a cell-free system based on washed trypanosome membranes (Masterson *et al.*, 1989, 1990; Doering *et al.*, 1989; Menon *et al.*, 1990a,b) have led to the delineation of the GPI precursor biosynthetic pathway (Doering *et al.*, 1990). The first step in this pathway is the addition of GlcNAc to PI to form GlcNAc-PI which is rapidly deacetylated to form GlcNH_2-PI. Three mannose residues are added serially from mannosyl phosphoryldolichol to form Man_3GlcNH_2-PI. The phosphoethanolamine moiety is then added from a membrane bound donor, recently identified as phosphatidylethanolamine (A.K.Menon, S.Mayor and R.T.Schwarz, personal communication), to form a species termed glycolipid A'. Finally, glycolipid A' undergoes a series of unusual fatty acid remodelling reactions to form the mature GPI precursor, which contains two myristic acid residues.

The phosphoethanolamine moiety provides a bridge between the glycolipid and the protein, and is therefore an indispensable component of the GPI anchor. Here we report that the serine esterase inhibitor, phenylmethanesulphonyl fluoride (PMSF) inhibits the incorporation of phosphoethanolamine into the GPI precursor, resulting in the accumulation of a Man_3GlcNH_2-PI intermediate. The implications of this for the study of GPI biosynthesis and function are discussed.

Results

Figure 1A shows how increasing PMSF concentrations affected GPI precursor biosynthesis in the cell-free system. In the absence of PMSF (lane 1) a number of previously characterized GPI intermediates (Masterson *et al.*, 1989, 1990) were labelled. Two of these intermediates (glycolipids A' and Θ') have a phosphoethanolamine group blocking their terminal mannose (Masterson *et al.*, 1989, 1990). Glycolipid A' only differs from glycolipid A (the GPI precursor) in its fatty acid composition; glycolipid Θ is the *lyso* form of glycolipid A' (Masterson *et al.*, 1990). At 0.25 mM PMSF (lane 4) incorporation of [^{3}H]mannose into glycolipids A' and Θ was almost abolished and label accumulated instead in a species co-chromatographing with Man_3GlcNH_2-PI (band III in Figure 1A; bands I and II are Man_1GlcNH_2-PI and Man_2GlcNH_2-PI respectively). The same effect could be achieved using diisopropyl fluorophosphate (1 mM; Figure 1B), whereas serine protease inhibitors such as 4-(amidinophenyl)methanesulphonyl fluoride (0.25 mM), 3,4 dichloroisocoumarin (0.05 mM), leupeptin (2 μg/ml), aprotinin (8 μg/ml), or *N*-tosyl-L-lysine chloromethyl ketone (0.1 mM) had no effect (data not shown). This putative Man_3GlcNH_2-PI glycolipid synthesized in the presence of PMSF was shown to be PI-PLC sensitive (Figure 1A, lanes

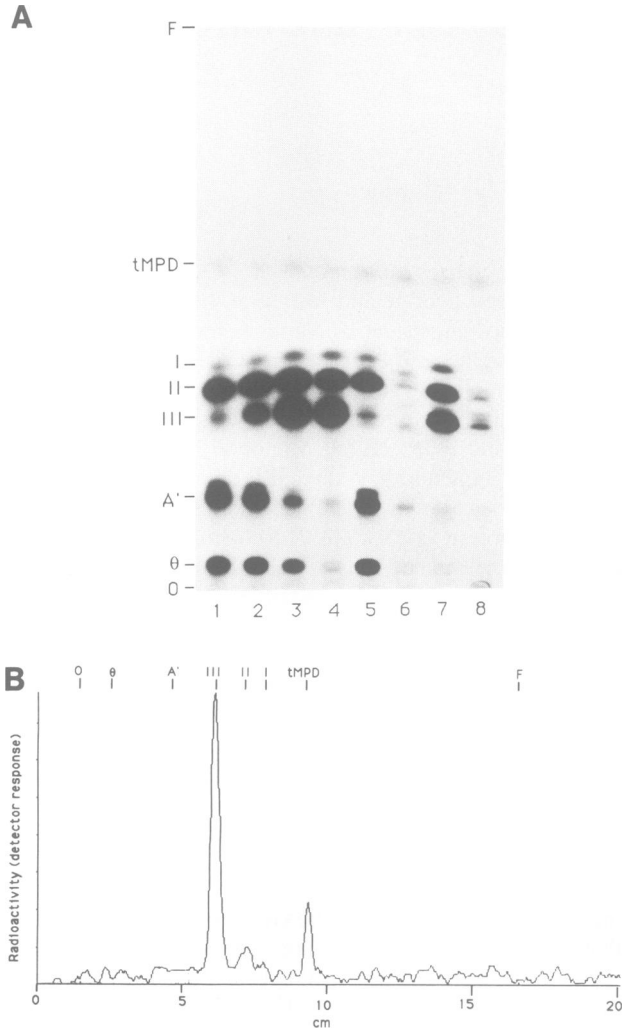

Fig. 1. A. Effect of PMSF on radiolabelling of GPI intermediates in the cell-free system. The cell-free system was incubated with GDP-[^{3}H]mannose in the absence (lane 1) or presence of 0.01, 0.05, or 0.25 mM PMSF (lanes 2–4). Samples identical to lane 1 (lanes 5 and 6) or lane 4 (lanes 7 and 8) were incubated with PI-PLC (lanes 6 and 8) or buffer alone (lanes 5 and 7) as described (Masterson *et al.*, 1989). Radioactivity lost in lanes 6 and 8 was recovered in the aqueous phase of the *n*-butanol/water partition performed prior to TLC, indicating release of the polar headgroup from the lipid. Each lane of the figure contains 3.5 × 10^6 cell equivalents of lipid. O = origin, F = solvent front. Other abbreviations to the left of the figure denote GPI intermediates (Masterson *et al.*, 1989, 1990; Menon *et al.*, 1990a). tMPD = trypanosomal mannosyl phosphoryldolichol; I, II, III = Man_1, Man_2, and Man_3GlcNH_2-PI respectively. **B.** Effect of diisopropyl fluorophosphate (DFP) on radiolabelling of GPI intermediates in the cell-free system. The cell-free system was incubated with GDP-[^{3}H]mannose in the presence of 1 mM DFP. The glycolipid fraction was analysed by TLC. Radioactivity was detected using a Raytest linear analyser. The positions of the origin, front, and various standards are indicated. Abbreviations as above.

7 and 8), confirming that it contains a PI phospholipid moiety. Furthermore, base hydrolysis, nitrous acid deamination and borohydride reduction generated a radiolabelled headgroup from this material which had an elution position on Dionex HPLC of 2.5 glucose units (Figure 2A) and on Bio-Gel P-4 gel filtration of 4.2 glucose units (data not shown). Authentic Manα1-2Manα1-6Manα1-4(2,5)-anhydromannitol (Man_3AHM) derived from VSG (Ferguson *et al.*, 1988) elutes at exactly these positions in the two chromatographic systems (Schneider *et al.*, 1990).

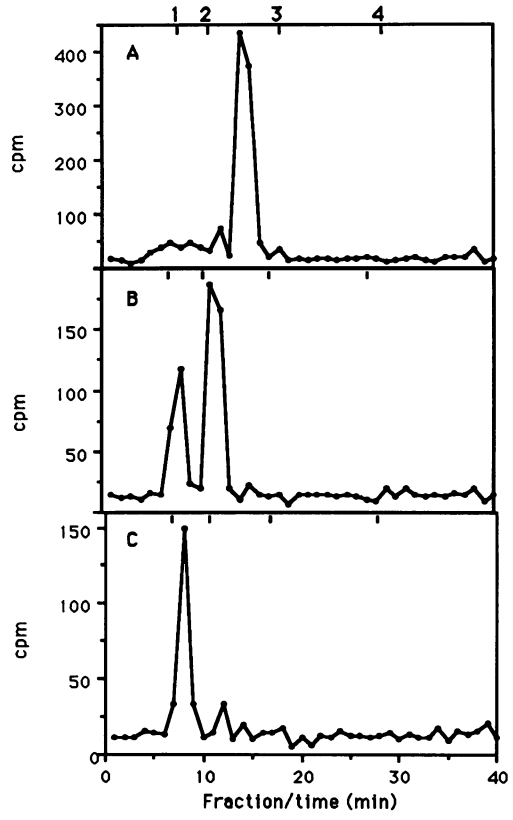

Fig. 2. Structural characterisation of the Man_3GlcNH_2-PI headgroup. **Panel A.** The putative Man_3GlcNH_2-PI generated in the cell-free system in the presence of PMSF was TLC-purified, deacylated, deaminated, and reduced. This radiolabelled glycan (80 000 c.p.m.) was analysed by Dionex HPLC. **Panel B.** To provide further evidence of identity, the 2.5 glucose unit peak from panel A was desalted and digested with *Aspergillus phoenicis* α-mannosidase (which is specific for Manα1-2Man glycosidic linkages). Dionex HPLC analysis now revealed peaks of 1.2 and 2.2 glucose units with twice as much label in the latter peak. These peaks co-eluted with authentic mannose and Manα1-6Manα1-4AHM (Man_2AHM) standards respectively. **Panel C.** The 2.2 glucose unit peak from panel B was desalted, digested with jack bean α-mannosidase (which has a broad specificity for α-linked mannose), and reanalysed by Dionex HPLC. All the radioactivity now eluted as free mannose at 1.2 glucose units. The numbers above the figure refer to the elution positions of the Glc_1 to Glc_4 dextran oligomer internal standards. Recoveries of radioactivity at each step were >75%.

Together with the exoglycosidase sequencing data (Figure 2), these data strongly suggest that the glycolipid structure is Manα1-2Manα1-6Manα1- 4$GlcNH_2$-PI. The abolition of labelling of glycolipids Θ and A′ and the accumulation of Man_3GlcNH_2-PI in the cell-free system suggest that PMSF blocks phosphoethanolamine addition.

Trypanosomes were biosynthetically labelled with [^{3}H]mannose and Figure 3 shows that PMSF also blocks phosphoethanolamine addition in living cells. In control cells (lanes 1–4) two major glycolipids were labelled. One of these is glycolipid A (the GPI precursor), the other is glycolipid C. Glycolipid C only differs from glycolipid A in having an extra acyl group in hydroxy-ester linkage to its inositol ring (Krakow *et al.*, 1989; Mayor *et al.*, 1990a); hence its higher R_f on TLC. In PMSF-treated cells the synthesis of glycolipid A is substantially inhibited, the synthesis of glycolipid C is abolished and there is a build-up of labelled material at and around band III (Figure 3, lanes 5–8). This latter material was shown to be PI-PLC

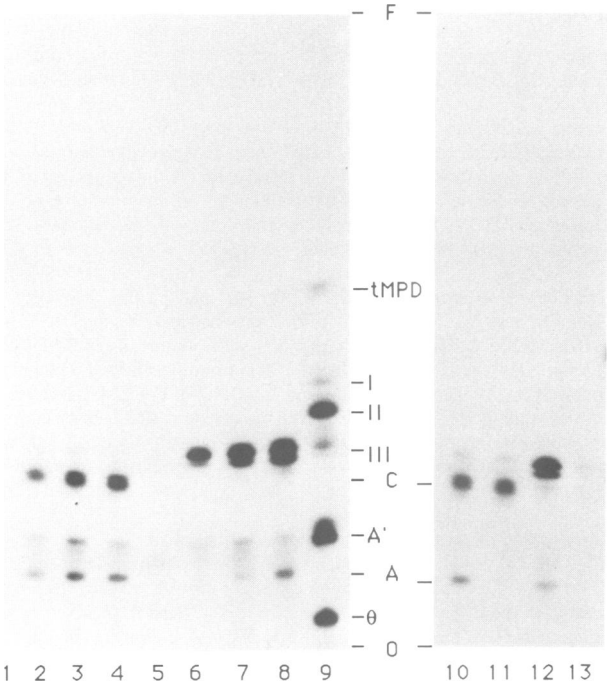

Fig. 3. Effect of PMSF on the labelling of trypanosomes with [^{3}H]mannose. Trypanosomes were metabolically labelled with [^{3}H]mannose in the absence (lanes 1–4) or presence (lanes 5–8) of 0.8 mM PMSF. Time points were taken at 0 (lanes 1 and 5), 5 min (lanes 2 and 6), 10 min (lanes 3 and 7) and 20 min (lanes 4 and 8). Glycolipid extracts were analysed by TLC as for Figure 1. Lanes 1–8 each contain 1.7 × 10^6 cell equivalents of lipid. For comparison, lane 9 contains lipid labelled using the cell-free system as Figure 1, lane 1. Extracts from cells labelled with [^{3}H]mannose in the absence (lanes 10 and 11) or presence of PMSF (lanes 12 and 13) were treated with PI-PLC (lanes 11 and 13) or with buffer alone (lanes 10 and 12). Radioactivity lost in lanes 11 and 13 was recovered in the aqueous phase of the *n*-butanol/water partition performed prior to TLC. Lanes 1–9 and 10–13 are from different TLCs. A and C refer to glycolipids A and C. Other abbreviations are as in Figure 1.

sensitive (Figure 3, lanes 12 and 13) and characterization of the glycolipid head groups gave identical results to those shown in Figure 2 (data not presented). Thus the Man_3GlcNH_2-PI synthesized in PMSF-treated cells appears to be more heterogeneous with respect to the lipid moiety than that synthesized in the cell free system (compare Figures 1 and 3). Fatty acid remodelling is known to occur during GPI biosynthesis in *T.brucei* and the heterogeneity observed in Figure 3 may result from incomplete remodelling of the acyl groups on the Man_3GlcNH_2-PI species. If so, this is the first observation of remodelling intermediates in intact trypanosomes. These unusual remodelling reactions are peculiar to *T.brucei* and may be targets for selective chemotherapy (Masterson *et al.*, 1990).

Samples of trypanosomes labelled with [^{3}H]mannose were also analysed by SDS–PAGE to determine the effect of PMSF on VSG biosynthesis (Figure 4). Treatment with endoglycosidase H caused a shift in apparent mol. wt of the labelled VSG from 59 kd to 57 kd, consistent with the removal of the single site oligomannose N-linked glycans described for this VSG (Zamze *et al.*, 1990). Quantitative removal of the N-linked glycans caused an 80% reduction in the amount of [^{3}H]mannose label found in the VSG band in both cases, with the residual radioactivity belonging to the protein-linked GPI anchor. This result indicates that

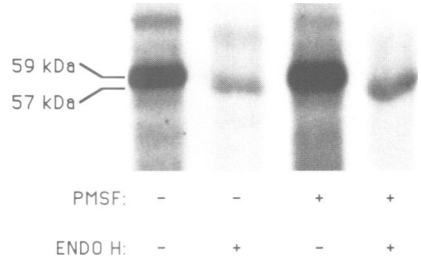

Fig. 4. Effect of PMSF on incorporation of [^{3}H]mannose into VSG GPI. Extracts of trypanosomes metabolically labelled with [^{3}H]mannose in the absence or presence of 0.8 mM PMSF were mock-treated or endoglycosidase H-treated as indicated, and analysed by SDS–PAGE on an 8% resolving gel. The figure shows the VSG region of the fluorographed gel. Each lane contains extract equivalent to 4 × 10^6 cells. Corroboration of the identity of the indicated 59 kd and 57 kd bands as VSG was obtained by immunoblotting of an identical gel with an anti MIT at 1.4 VSG polyclonal antiserum (not shown).

despite the significant reduction in GPI precursor biosynthesis caused by PMSF (Figure 3) there is still sufficient precursor in these cells to allow efficient transfer of GPI to newly synthesized VSG. The marked reduction in glycolipid C synthesis (Figure 3, lanes 5–9) in these same PMSF-treated cells, could suggest that acylation of the inositol ring of the precursor is not essential for either GPI precursor assembly or GPI addition to protein. However, these data do not rule out glycolipid C as an intermediate in either of these processes. The function of glycolipid C in bloodstream form trypanosomes therefore remains obscure.

Discussion

In both living cells and a trypanosome cell-free system, addition of PMSF results in the accumulation of a GPI species that appears by a number of criteria to be Manα1-2Manα1-6Manα1-4$GlcNH_2$-PI (Man_3GlcNH_2-PI). These criteria include TLC migration, PI-PLC sensitivity, Dionex HPLC and Biogel P-4 chromatography, and exoglycosidase sequencing. One possible explanation for the accumulation of Man_3GlcNH_2-PI in living cells is that PMSF causes a general depression of cellular metabolism. However trypanosomes seemed unaffected by PMSF, as judged by their motility and their ability to incorporate [^{3}H]mannose (Figure 4) and [^{35}S]methionine (data not shown) into protein. PMSF does not block [^{3}H]ethanolamine incorporation into phosphatidylethanolamine in trypanosomes (data not shown) ruling out inhibition of donor synthesis as an explanation. We therefore propose that PMSF interferes with GPI biosynthesis by inhibiting phosphoethanolamine transfer to Man_3GlcNH_2-PI from phosphatidylethanolamine.

Inhibition of the phosphoethanolamine transferase by PMSF suggests that this enzyme might belong to the general class of enzymes with an active-site serine. PMSF is known to sulphonylate the hydroxyl group of the active-site serine of chymotrypsin (Gold, 1965, 1967), the best characterized enzyme of this class. Further support for this suggestion comes from the observed accumulation of Man_3GlcNH_2-PI in the cell-free system in the presence of di-isopropyl fluorophosphate, a reagent which specifically phosphorylates activated serines. In contrast, several protease inhibitors had no effect at high concentration, ruling out the possibility that

PMSF inhibits proteolytic activation of the phosphoethanolamine transferase. Given these observations, we propose a two-step reaction mechanism for phosphotethanolamine transfer in GPI biosynthesis. In the first step, the active serine of the transferase would react with phosphatidylethanolamine forming an enzyme-phosphoethanolamine intermediate and releasing diglyceride. In the second step, the phosphoethanolamine would be transferred onto the C-6 hydroxyl group of the terminal mannose of Man_3GlcNH_2-PI, with the enzyme being released for a further round of transfer.

Phosphoethanolamine incorporation into GPI species is inhibited some 90% by PMSF in living cells (Figure 3). This is sufficient to allow us for the first time to generate radiolabelled Man_3GlcNH_2-PI to use as a substrate for the identification and purification of the phosphoethanolamine transferase. Similarly, digestion of this intermediate with α-mannosidases can generate substrates for studying the earlier reactions of the pathway. That the inhibition by PMSF was insufficient to inhibit GPI addition to protein (Figure 4) is disappointing. However, with knowledge of both the acceptor and donor structure, and the general class of enzyme involved, design of a more potent and selective inhibitor of the phosphoethanolamine transfer step becomes a real possibility. The value of such compounds for both the study of GPI function and possibly as novel trypanocidal agents is apparent.

Materials and methods

Preparation of trypanosome cell-free system

Lysates of trypanosomes (variant MITat 1.4) were prepared and stored as described previously (Masterson *et al.*, 1989), except that the incubation with tunicamycin prior to hypotonic lysis was omitted. Membranes for cell-free incorporation experiments were prepared by thawing an aliquot (1 ml) of lysate and washing as described (Masterson *et al.*, 1989). Membranes were finally resuspended in 0.5 ml 100 mM Na-HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 2.5 μg/ml tunicamycin, 0.2 mM *N*-tosyl-L-lysine chloromethyl ketone, 2 μg/ml leupeptin.

Radiolabelling of glycolipids in the cell-free system

0.5 μCi of GDP-[3,4-^{3}H]mannose (16.8 Ci/mmol; New England Nuclear) was dried and redissolved in 15 μl of 2 mM UDP *N*-acetyl glucosamine +/− inhibitor (for final concentrations see text and figure legends). To this was added 15 μl membranes prepared as above. After incubating 15 min at 37°C, the reaction was stopped by the addition of 0.4 ml chloroform/methanol (1:1). Processing of the extract for TLC analysis of the glycolipid fraction was as previously described (Masterson *et al.*, 1989). In such experiments the proportion of radiolabel incorporated into glycolipid was usually 20−25%.

Metabolic labelling of trypanosomes with [^{3}H]mannose

MIT at 1.4 trypanosomes from the buffy coat of infected rat blood were washed and suspended at 2.5 × 10^7/ml in sugar label medium (glucose-free RPMI 1640 buffered to pH 7.4 with 25 mM Na-HEPES and supplemented with 3 mg/ml glycerol, 10 mg/ml bovine serum albumin, 12 μg/ml adenosine, and 1 μg/ml catalase). PMSF was added at this stage from a stock solution (80 mM in propan-2-ol). Control cells had the same volume of propan-2-ol added. Following a 10 min incubation in a 37°C shaking water bath, [2-^{3}H]mannose (18.5 Ci/mmol; Amersham) was added in 25 μl sugar label medium to give a final concentration of 50 μCi/ml. At 0, 5, 10 and 20 min 0.1 ml aliquots were removed and added to 1 ml ice-cold sugar label medium. The trypanosomes were pelleted in an Eppendorf microfuge (30 s, 14 000 r.p.m.) and extracted with 0.25 ml chloroform/methanol/water (1:1:0.3). This extract was processed for TLC as described (Krakow *et al.*, 1986). In all such experiments incorporation of [^{3}H]mannose into VSG was linear throughout the time course of the experiment (data not shown).

Endoglycosidase H treatment of [^{3}H]mannose labelled extracts

Trypanosomes metabolically labelled with [^{3}H]mannose for 30 min in the presence or absence of 0.8 mM PMSF were lysed at 6 × 10^8/ml in 0.1 M ammonium acetate pH 5.5, 0.3% w/v SDS, 2% v/v 2-mercaptoethanol (100°C, 5 min). After cooling to room temperature, 2 vol of 0.1 M ammonium acetate pH 5.5, 3% w/v Triton-X 100, 0.75 mM PMSF, 0.15 mM TLCK, 0.03% NaN_3 were added. Extracts were digested with or without endoglycosidase H (Boehringer) at 66 mU/ml for 16 h at 37°C. The digest was terminated by the addition of an equal volume of 2× concentrated SDS−PAGE sample buffer (5 min, 100°C). Samples were analysed by SDS−PAGE and fluorography.

Thin Layer Chromatography

Samples were applied in 10 μl chloroform/methanol/water (1:1:0.3) to activated (100°C, 30 min) silica gel 60 HPTLC aluminium-backed sheets (Merck). TLC plates were developed in chloroform/methanol/1 M ammonium acetate/13 M ammonia/water (180:140:9:9:23). For TLC purification, radiolabelled glycolipids were first detected using a RITA TLC analyser (Raytest), then the relevant sections of the TLC were scraped and extracted with 0.4 ml methanol/pyridine/water 2:1:1). Fluorography was as described (Krakow *et al.*, 1986).

Generation of deaminated and reduced glycans

50−100 000 c.p.m. of TLC-purified glycolipid was deacylated in 50 μl 1:1 methanol/35% aqueous ammonia (37°C, 16 h). After drying, the sample was dissolved in 10 μl 0.3 M Na acetate, pH 4.0 and deaminated by the addition of 10 μl freshly prepared 0.5 M $NaNO_2$. Following deamination (3 h, room temperature), the sample was reduced (16 h, 4°C) by adding 5 μl 0.4 M boric acid, 18 μl of 1 M NaOH and 40 μl of 1 M $NaBH_4$ (in 0.1 M NaOH). Excess reductant was destroyed by adding 50 μl 1 M acetic acid. The sample was desalted by passage through a 0.3 ml column of AG50 × 12(H^+) and drying twice from 5% acetic acid in methanol and twice from methanol. Radiolabel was quantitatively recovered in the desalted material.

Dionex carbohydrate HPLC

[^{3}H]mannose labelled glycan samples were dissolved in 25 μl water containing 150 μg of mixed glucose oligomers from a dextran partial acid hydrolysate. Separation was achieved using a Dionex Bio-LC HPLC system equipped with a Carbopak anion exchange column, pulsed amperiometric detector (PAD) and an anion micro-membrane suppressor. Radioactive components were detected with a Ramona on-line radioactivity monitor (Raytest Instruments) and by liquid scintillation counting of aliquots of 1 min fractions. The glucose oligomer internal standards were detected by PAD. The elution positions of the radioactive components are expressed in glucose units by linear interpolation of their elution positions between adjacent glucose oligomers (Schneider *et al.*, 1990). These values have no precise meaning but are an absolute chromatographic property, unlike retention time. The buffer program used was a linear gradient from 12.5 mM to 50 mM sodium acetate in 150 mM sodium hydroxide over 50 min followed by a wash cycle of 20 min using 250 mM sodium acetate in 150 mM sodium hydroxide. The flow rate was 0.6 ml/min.

Exoglycosidase digestions

Samples isolated from Dionex HPLC were desalted by passage through 0.2 ml AG50 × 12(H^+) over 0.2 ml AG3 × 4(OH^-) over 0.2 ml Sephadex QAE-A25 and elution with water. *Aspergillus phoenicis* α-mannosidase (Oxford Glycosystems) digests were performed for 16 h at 37°C with 20 μl of 2 μg/ml enzyme in 0.1 M sodium acetate pH 5.0. Jack bean α-mannosidase (Boehringer) digests were performed for 16 h at 37°C in 20 μl of 25 U/ml enzyme in 0.1 M sodium acetate pH 5.0. All enzyme digests were terminated by heating to 100°C for 5 min and desalted by passage through 0.2 ml AG50 × 12(H^+) followed by evaporation.

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References

Cross,G.A.M. (1990) *Annu. Rev. Cell Biol.*, **6**, 1−39.

Doering,T.L., Masterson,W.J., Hart,G.W. and Englund,P.T. (1989) *J. Biol. Chem.*, **264**, 11168−11173.

Doering,T.L., Masterson,W.J., Hart,G.W. and Englund,P.T. (1990) *J. Biol. Chem.*, **265**, 611–614.
Dotti,C.G., Parton,R.G. and Simons,K. (1991) *Nature*, **349**, 158–161.
Ferguson,M.A.J., Homans,S.W., Dwek,R.A. and Rademacher,T.W. (1988) *Science*, **239**, 753–759.
Ferguson,M.A.J. and Williams,A.F. (1988) *Annu. Rev. Biochem.*, **57**, 285–320.
Ferguson,M.A.J. and Homans,S.W. (1989) In McAdam,K.P. (ed.), *New Strategies in Parasitology*, Churchill Livingston, Edinburgh, pp. 121–140.
Gold,A.M. (1965) *Biochemistry*, **4**, 897–901.
Gold,A.M. (1967) *Methods Enzymol.*, **11**, 706–711.
Homans,S.W., Ferguson,M.A.J., Dwek,R.A. and Rademacher,T.W. (1988) *Nature*, **333**, 269–272.
Krakow,J.L., Hereld,D., Bangs,J.D., Hart,G.W. and Englund,P.T. (1986) *J. Biol. Chem.*, **261**, 12147–12153.
Krakow,J.L., Doering,T.L., Masterson,W.J., Hart,G.W. and Englund,P.T. (1989) *Mol. Biochem. Parasitol.*, **36**, 263–270.
Lisanti,M.P., Caras,I.W., Davitz,M.A. and Rodriguez-Boulan,E. (1989) *J. Cell. Biol.*, **109**, 2145–2156.
Low,M.G. (1989) *Biochim. Biophys. Acta*, **988**, 427–454.
Masterson,W.J., Doering,T.L., Hart,G.W. and Englund,P.T. (1989) *Cell*, **56**, 793–800.
Masterson,W.J., Raper,J., Doering,T.L., Hart,G.W. and Englund,P.T. (1990) *Cell*, **62**, 73–80.
Mayor,S., Menon,A.K. and Cross,G.A.M. (1990a) *J. Biol. Chem.*, **265**, 6174–6181.
Mayor,S., Menon,A.K., Cross,G.A.M., Ferguson,M.A.J., Dwek,R.A. and Rademacher,T.W. (1990b) *J. Biol. Chem.*, **265**, 6164–6173.
Menon,A.K., Mayor,S. and Schwarz,R.T. (1990a) *EMBO J.*, **9**, 4249–4258.
Menon,A.K., Schwarz,R.T., Mayor,S. and Cross,G.A.M. (1990b) *J. Biol. Chem.*, **265**, 9033–9042.
Roberts,W.L., Santikarn,S., Reinhold,V.N., and Rosenberry,T.L. (1988) *J. Biol. Chem.*, **263**, 18776–18784.
Robinson,P.J., Millrain,M., Antoniou,J., Simpson,E. and Mellor,A.L. (1989) *Nature*, **342**, 85–87.
Rothberg,K.G., Ying,Y., Kolhouse,J.F., Kamen,B.A. and Anderson,R.G.W. (1990) *J. Cell Biol.*, **110**, 637–649.
Schneider,P., Ferguson,M.A.J., McConville,M.J., Mehlert,A., Homans,S.W. and Bordier,C. (1990) *J. Biol. Chem.*, **265**, 16955–16964.
Thomas,J.R., Dwek,R.A. and Rademacher,T.W. (1990) *Biochemistry*, **29**, 5413–5422.
Zamze,S.E., Wooten,E.W., Ashford,D.A., Ferguson,M.A.J., Dwek,R.A. and Rademacher,T.W. (1990) *Eur. J. Biochem.*, **187**, 657–663.

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