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Mannosamine, a novel inhibitor of glycosyl-phosphatidylinositol incorporation into proteins

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Mannosamine (2-amino-2-deoxy D-mannose) is shown here to block the incorporation of glycosyl-phosphatidylinositol (GPI) into GPI-anchored proteins. The amino sugar drastically reduced the surface expression of a recombinant GPI-anchored protein in polarized MDCK cells, converted this apical membrane-bound protein to an unpolarized secretory product and blocked the expression of endogenous GPI-anchored proteins. Furthermore, it specifically inhibited the incorporation of [^{3}H]ethanolamine (a GPI component) into mammalian and trypanosomal GPI-anchored proteins and into a well characterized GPI-lipid of *Trypanosoma brucei*. These results suggest that mannosamine converted an apical GPI-anchored protein to a non-polarized secretory product by depleting transfer competent GPI-precursor lipids. Our inhibitor studies provide new independent evidence for the apical targeting role of GPI in polarized epithelia and open the way towards a greater understanding of the functional role of GPI in membrane trafficking and cell regulation.
Key words: glycosylation inhibitor/MDCK cells/membrane trafficking/polarized sorting

Introduction

An increasing number of membrane glycoproteins are found anchored to the cell surface via a C-terminal glycophospholipid (GPI; glycosyl-phosphatidylinositol), instead of by conventional transmembrane hydrophobic peptide sequences (Low and Saltiel, 1988; Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Lisanti *et al.*, 1990c; Thomas *et al.*, 1990). Analyses of the GPI-anchors of several proteins indicate that the anchors contain a conserved core glycan structure, ethanolamine-phosphate-6 Manα1-2 Manα1-6 Manα1-4 GlcNα1-6 Inositol (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Schneider *et al.*, 1990). Studies using trypanosome cell-free preparations (Doering *et al.*, 1989, 1990; Masterson *et al.*, 1989; Menon *et al.*, 1990b) indicate that GPI biosynthesis proceeds by sequential glycosylation of phosphatidylinositol (PI). First, *N*-acetylglucosamine is added from UDP-GlcNAc to form GlcNAc-PI. GlcNAc-PI is then deacetylated to form GlcN-PI, three mannoses are added from dolichol-P-mannose (Dol-P-Man; Menon *et al.*, 1990a) and the assembly is completed by adding the terminal ethanolamine-phosphate group. Transfer of the completed GPI to a protein acceptor occurs rapidly (Bangs *et al.*, 1985, 1986; Ferguson *et al.*, 1986), probably via a transpeptidation reaction that removes a C-terminal hydrophobic peptide that acts both as the signal for GPI-attachment and as a transient membrane anchor (Caras *et al.*, 1987, 1989; Moran *et al.*, 1991).

Although the pathway for synthesis of GPI-precursor lipids and the signals directing their attachment to protein have been characterized in some detail, little is known about the function of GPI. Recent studies have proposed a role for the anchor in protein trafficking (Rodriguez-Boulan and Nelson, 1989; Lisanti and Rodriguez-Boulan, 1990, 1991). Endogenous and exogenous GPI-anchored proteins expressed in the polarized renal epithelial line MDCK were apically localized (Brown *et al.*, 1989; Lisanti *et al.*, 1989, 1990b; Wilson *et al.*, 1990; Powell *et al.* 1991), as were chimeric glycoproteins containing the ectodomains of either basolateral antigens or of secretory proteins linked to GPI via fusion with sequences specifying GPI-attachment (Brown *et al.*, 1989; Lisanti *et al.*, 1989, 1991). This apical distribution results from intracellular sorting and vectorial delivery and appears to be tight junction-independent (Lisanti *et al.*, 1990a). A similar sorting mechanism appears to operate in neurons, as several GPI-anchored proteins (5′ nucleotidase, acetylcholinesterase, neuronal cell surface protein F3, and Thy-1) are localized to axons and nerve terminal regions while excluded from cell bodies and dendrites (reviewed in Lisanti and Rodriguez-Boulan, 1990, 1991; Dotti *et al.*, 1991).

Studies on the functional significance of the anchor in cell regulation are currently hampered by the lack of inhibitors of GPI synthesis or attachment. *N*-glycosylation inhibitors have been particularly useful in defining the role of these carbohydrates in a variety of cellular processes (Sly, 1982; Elbein, 1987; Kornfeld, 1990). Unfortunately, the only inhibitors of GPI synthesis available (e.g. 2-fluoro 2-deoxy D-glucose and amphomycin, both inhibitors of Dol-P-Man synthesis; Schwarz *et al.*, 1989; Menon *et al.*, 1990a) are also potent inhibitors of protein synthesis (fluoroglucose) or are only active in cell-free extracts (amphomycin). Recently PMSF was found to inhibit the addition of ethanolamine to GPI, but paradoxically it did not prevent GPI-anchoring of the protein product (Masterson and Ferguson, 1991).

We report here that an amino sugar, mannosamine, drastically blocks the synthesis of GPI and the incorporation of GPI glycans into GPI-anchored proteins without affecting the synthesis of the protein component. A GPI-anchored protein was converted from an apical plasma membrane protein to an unpolarized secretory product, supporting the apical targeting role of GPI (Lisanti and Rodriguez-Boulan, 1990, 1991). As mannosamine is also active in *Trypanosoma brucei*, where it specifically blocks the synthesis of GPI

precursors, it may prove useful for the treatment of parasitic diseases in which the main parasite coat protein is a GPI-anchored protein [e.g. African trypanosomiasis, Chaga's disease, leishmaniasis, malaria, schistosomiasis, and toxoplasmosis (Low, 1989)].

Results

The amino sugar mannosamine biosynthetically prevents the formation of alpha 1,2 mannose linkages in N-linked glycans, but does not affect their transfer to protein and processing from high mannose to hybrid and complex structures (Pan and Elbein, 1985). As a single alpha 1,2 mannose linkage is present in the conserved core glycan of the GPI-anchor (Figure 1; Ferguson and Williams, 1988; Low and Saltiel, 1988; Low, 1989), we suspected that mannosamine might act as an inhibitor of GPI-attachment. To test this hypothesis, we used MDCK cells permanently transfected with gD-1-DAF, a recombinant molecule containing the ectodomain of a basolateral viral glycoprotein (gD-1) fused to the DAF signal for GPI-attachment (Caras *et al.*, 1987). We have previously shown that this fusion protein is GPI-anchored and expressed on the apical surface of polarized MDCK cells (Lisanti *et al.*, 1989).

Mannosamine inhibits the surface expression of gD-1-DAF

Mutant cell lines that fail to incorporate GPI into protein show, characteristically, a drastic decrease in surface expression and accumulation of the protein portion in the secretory pathway. Mannosamine had a similar effect on the cellular distribution of gD-1-DAF, as studied by immunofluorescence microscopy (Figure 2, upper and middle panels). While in control cells, gD-1-DAF was localized predominantly on the apical surface (Lisanti *et al.*, 1989), in mannosamine-treated cells the gD-1-DAF distribution was dramatically altered to a reticular and peri-nuclear localization, reminiscent of the endoplasmic reticulum and the Golgi apparatus. This localization pattern was identical to that observed with a secretory form of the molecule, truncated gD-1 (which lacks a signal for GPI-attachment; Figure 2, lower panel), suggesting that a GPI-anchored protein had been transformed into a secretory protein in the presence of mannosamine.

If the effect of mannosamine was indeed to block the addition of GPI, the surface expression of gD-1-DAF would be inhibited in the presence of the amino sugar. To confirm the decreased surface expression of gD-1-DAF in mannosamine-treated cells observed by immunofluorescence (Figure 2), we used a surface biotinylation procedure described in our laboratory (Lisanti *et al.*, 1988; Sargiacomo *et al.*, 1989). As shown in Figure 3a, mannosamine inhibited the expression of gD-1-DAF on both the apical and the basolateral surfaces. Mannosamine also inhibited the delivery of intracellular gD-1-DAF to the cell surface (Figure 3b), as measured by a biotin targeting assay (Lisanti *et al.*, 1990a). In both cases, the residual fraction present on the cell surface was GPI-anchored (not shown) and apically polarized. These results indicate that mannosamine did not cause mis-sorting of the residual fraction that was GPI-anchored. In addition, the patterns of endogenous apical and basolateral proteins remained distinct after mannosamine treatment (not shown).

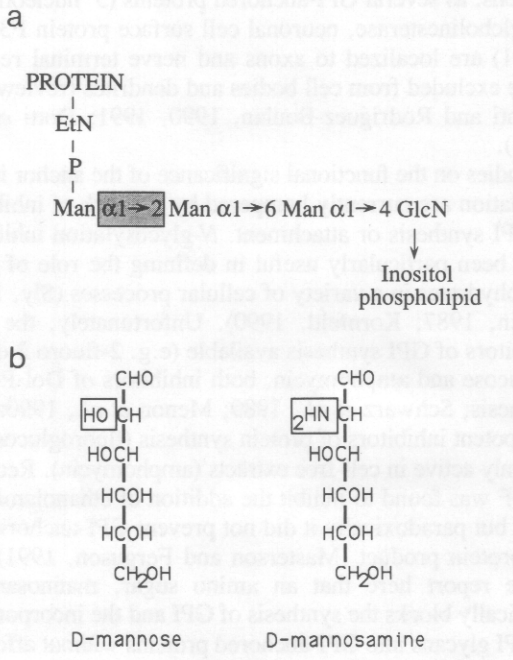

Fig. 1. Structure of the conserved core of the GPI-glycan and mannosamine. (**a**)The alpha 1.2 linkage of the conserved GPI-glycan is highlighted to illustrate the the expected site of mannosamine action. (**b**) Fischer projections of D-mannose and D-mannosamine. The C-2 position is boxed to indicate where the hydroxyl group of mannose is replaced by an amino group in mannosamine.

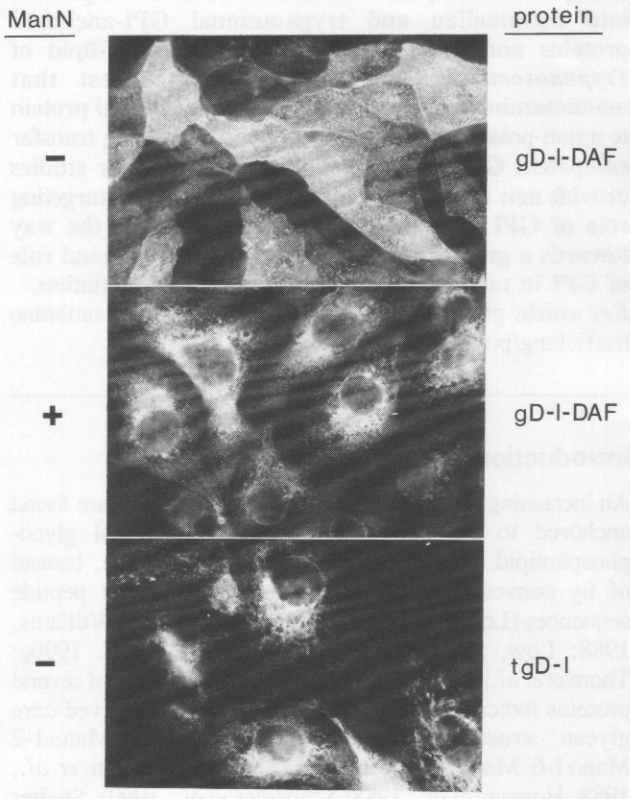

Fig. 2. Mannosamine alters the cellular distribution of gD-1-DAF. MDCK cells expressing gD-1-DAF (upper and middle panels) or truncated gD-1 (tgD-1; lower panel) were fixed, permeabilized and immunostained with a monoclonal antibody to gD-1. Bound IgG were visualized with the appropriate rhodamine-conjugated secondary antibody. gD-1-DAF expressing cells were mannosamine treated (+, middle; 10 mM) or left untreated (−, upper)

Mannosamine converts apical gD-1-DAF to a non-polarized secretory protein

GPI-anchoring confers proteins with hydrophobic properties that can be detected by their partitioning into the detergent phases of Triton X-114 extracts (Low, 1989). Removal of the hydrophobic diacylglycerol moiety of GPI by PI-PLC can be conveniently monitored by the change in the biophysical properties of the protein, which then partitions with the aqueous phase in the absence of an intact GPI-anchor (Lisanti *et al.*, 1988). Accordingly, we determined the effect of mannosamine on the hydrophobic properties and on the biosynthesis and processing of gD-1-DAF by MDCK cells.

Consistent with its anchoring via GPI, gD-1-DAF partitions with the detergent phase of Triton X-114 cell extracts and is completely sensitive to the action of PI-PLC (Figure 4a). Two hydrophobic forms of the protein are detected, a precursor 40 kd form and a mature 45−50 kd glycosylated form (Figure 4b; Lisanti *et al.*, 1989). The mature form is located at the cell surface and is apically polarized, consistent with the postulated role of GPI as an apical targeting signal (Lisanti and Rodriguez-Boulan, 1990). No gD-1-DAF is detected in the aqueous phase of the cell extracts; however, a third hydrophilic form (35 kd) immunoreactive with gD-1 antibodies, was detected in the medium of control cells (Figure 4b). Several experiments indicated that this form of gD-1-DAF, 10 kd smaller than the mature form, originated from the proteolytic processing of the 45−50 kd form at the cell surface by an endogenous protease and was, therefore, predominantly found in the apical medium (Figure 4b; not shown).*

Biochemical experiments confirmed that mannosamine addition transformed gD-1-DAF into a secretory product. In mannosamine-treated MDCK cells, the major cell-associated form of immunoprecipitable gD-1-DAF was a hydrophilic molecule of 38−40 kd, instead of the 40 and 45−50 kd membrane-bound forms observed in control cells (Figure 5a). In the medium of mannosamine-treated cells, the 35 kd proteolytic product of gD-1-DAF was replaced by a 43 kd secretory form of gD-1-DAF, with electrophoretic mobility identical to truncated gD-1 that lacks a GPI-attachment signal (Figure 5b, see also Lisanti *et al.*, 1989). This secretory form of gD-1-DAF, unlike the 35 kd proteolytic product, was not polarized (Figure 5b). Residual amounts of the 35 kd form were detected in mannosamine-treated cells and were present in an apically polarized distribution, further indicating that mannosamine did not affect the tightness of the monolayer. In a previous paper, we have shown that truncated gD-1 was secreted either without polarity or basolaterally, depending on whether the expression level was high or low, respectively (Lisanti *et al.*, 1989). As GPI-anchoring is believed to act as an intracellular targeting signal for apical transport, it appears that in mannosamine-treated cells the GPI-anchor is either not added or removed prior to the site of apical sorting (the trans-Golgi network, TGN).

To determine whether the conversion of gD-1-DAF from a membrane-bound hydrophobic form to a hydrophilic form was dependent on a block in the attachment of GPI, we measured the incorporation of [^{3}H]ethanolamine, a component of the GPI anchor, into immunoprecipitable gD-1-DAF. Both mannosamine-induced forms of gD-1-DAF incorporated little or no [^{3}H]ethanolamine (Figure 6). Since ethanolamine cannot be removed by phospholipase cleavage (Low and Saltiel, 1988; Low, 1989), these results discard the possibility that the appearance of these forms might be due to activation of an intracellular phospholipase. Instead, they are consistent with a block in the addition of the anchor induced by mannosamine. No changes in the biosynthesis of gD-1-DAF were observed after treatment with glucosamine (not shown), another amino sugar that acts as a glycosylation inhibitor via a different mechanism (Pan and Elbein, 1982).

Pulse−chase experiments were undertaken to establish a precursor−product relationship between the different mannosamine-induced forms of gD-1-DAF (Figure 7a). Three forms were detected: a 41−43 kd hydrophobic precursor at time 0; a 38−40 kd hydrophilic precursor at 1 h of chase; and a 43 kd secretory product at 3 h of chase. From these results, it appears that mannosamine induces a transient hydrophobic precursor ($t_{1/2}$ = 1 h) that is cleaved to a hydrophilic form and later secreted. The mannosamine-

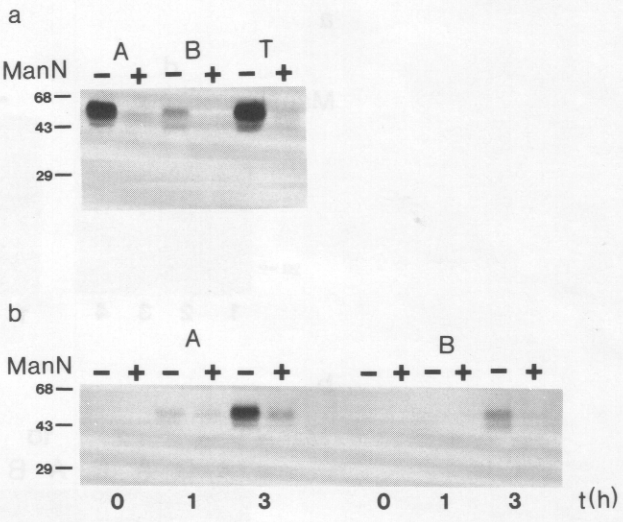

Fig. 3. Mannosamine inhibits cell-surface expression of gD-1-DAF. (**a**) Filter-grown monolayers were cultured overnight in the presence (+) or absence (−) of mannosamine (ManN; 10 mM) and then subjected to biotinylation at the apical (A), basolateral (B) or both surfaces (total, T) and immunoprecipitated. (**b**) Filter-grown monolayers were preincubated in glucose-free medium for 1 h in the presence (+) or absence (−) of mannosamine (ManN; 10 mM). Cell surface amino groups were quenched at 4°C and monolayers recultured at 37°C for various times (0, 1 and 3 h). Newly arriving amino groups were detected by domain selective biotinylation (A, apical; B, basolateral), immunoprecipitation and blotting with radio-labeled streptavidin. 'Splitting' of the band corresponding to the 45−50 kd form of gD-1-DAF was often observed and is caused by unlabeled comigrating IgG heavy chain used for immunoprecipitation.

* MDCK cells were subjected to surface biotinylation and recultured for various times to study the degradation of cell surface gD-1-DAF. The 45−50 kd cell-associated form was released to the apical medium as the 35 kd soluble form with a $t_{1/2}$ of 8 h, independently of serum factors. Several serine protease inhibitors prevented apical release by 70−90% (leupeptin, antipain, aprotinin, soybean trypsin inhibitor) and 50% (chymostatin), while other inhibitors specific for aspartyl proteases (pepstatin), cysteine proteases (E-64) and lysosomal proteases (chloroquine) had no effect. These results suggest that a trypsin-like enzyme was responsible for the cleavage event. A cell surface trypsin-like protease in hepatocytes (Tanaka *et al.*, 1986) posseses the same differential sensitivity to protease inhibitors as we have observed for the release of gD-1-DAF. Cleavage of gD-1-DAF at a single site that conforms to the specificity of the hepatic enzyme (leu−arg) would yield a fragment of the predicted size that we observe experimentally (residues 1−221).

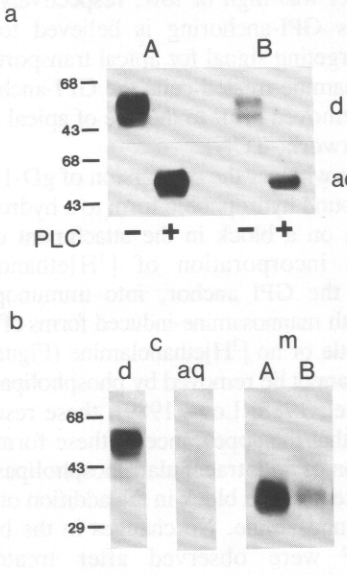

Fig. 4. PI-PLC sensitivity and hydrophobicity of the different forms of gD-1-DAF. (**a**) Filter-grown monolayers were biotinylated apically (A) or basolaterally (B) and extracted with the detergent Triton X-114. Detergent phases were collected and treated with PI-PLC (+ or −). Both detergent phases (d) and aqueous phases (aq) resulting after PI-PLC treatment were immunoprecipitated. Biotinylated proteins were detected by blotting with radiolabeled streptavidin. (**b**) Filter-grown monolayers were metabolically labeled overnight with ^{35}S-labeled amino acids. Cells (c) and media (m) were collected and extracted with the detergent Triton X-114. Detergent (d) and aqueous (aq) phases of cell extracts, as well as samples of apical (A) and basolateral (B) media, were subjected to immunoprecipitation. Metabolically labeled proteins were detected by fluorography.

induced precursor is slightly larger than the precusor seen in untreated control cells (1−3 kd). As gD-1-DAF contains a C-terminal GPI-attachment signal, 28 amino acids of which are removed prior to replacement with GPI (Moran *et al.*, 1991), a hydrophobic precursor protein would be expected to be 1−3 kd larger than the proteolytically processed form.

To test whether this precursor represents the C-terminally uncleaved translation product of the gD-1-DAF mRNA, we assayed its susceptibility to PI-PLC. Unfortunately, it was degraded under the conditions of our PI-PLC assay (Figure 7b), even when four different classes of protease inhibitors were included during the incubation (see Materials and methods). The normal precursor was, however, completely sensitive to PI-PLC treatment (Figure 7b). The instability of a PLC-resistant hydrophobic precursor may explain why different groups have reported contradictory results on its detectability in mutant cells defective in Dol-P-Man synthesis and GPI-attachment (Conzelmann *et al.*, 1986; Fatemi and Tartakoff, 1986). Nonetheless, since removal of the hydrophobic C-terminal signal sequence and addition of the GPI-anchor occurs rapidly (within 1−2 min of synthesis) (Bangs *et al.*, 1985; Ferguson *et al.*, 1986; Conzelmann *et al.*, 1987), the observation of a larger, hydrophobic, transient and unstable mannosamine-induced precursor of gD-1-DAF is consistent with it being the C-terminally uncleaved translation product of the gD-1-DAF mRNA, the intermediate just preceding cleavage and the attachment of GPI. Similarly, a candidate protein precursor of larger size appears transiently in the processing of alkaline phosphatase in JEG-3 cells (Takami *et al.*, 1988)

Taken together, all of the above results are consistent with mannosamine converting gD-1-DAF from an apical GPI-

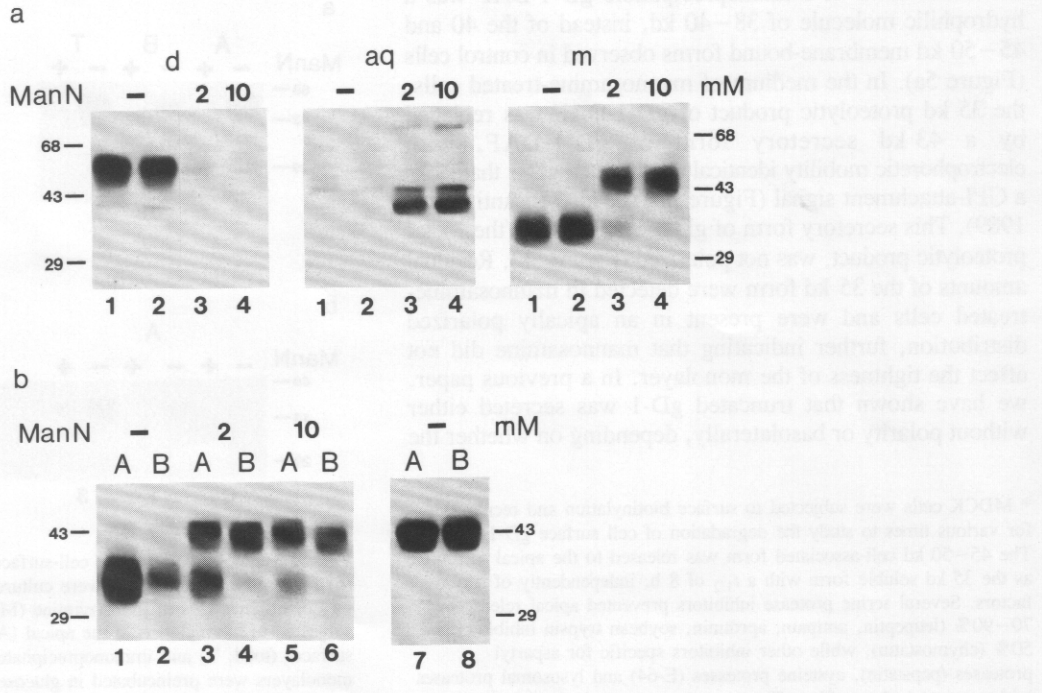

Fig. 5. Mannosamine alters the biosynthetic processing and leads to non-polarized secretion of a hydrophilic form of gD-1-DAF. (**a**) Plastic-grown cells were metabolically-labeled overnight with ^{35}S-labeled amino acids in the absence (−) or presence (2 and 10 mM) of mannosamine (ManN). Cell extracts were partitioned into detergent (d) and aqueous (aq) phases. Cell extracts and media (m) were then immunoprecipitated. Lanes 1 and 2: untreated controls; lane 3: treated with 2 mM mannosamine; lane 4: treated with 10 mM mannosamine. (**b**) Filter-grown MDCK cells expressing gD-1-DAF (lanes 1−6) or truncated gD-1 (lanes 7 and 8) were metabolically labeled overnight and the resulting media samples (A, apical; B, basolateral) were immunoprecipitated. Lanes 1 and 2: untreated controls; lanes 3 and 4: treated with 2 mM mannosamine; lanes 5 and 6: treated with 10 mM mannosamine.

anchored protein to a non-polarized secreted protein by preventing the incorporation of GPI. This effect of mannosamine is not due to inhibition of *N*-glycosylation, since mannosamine does not affect the transfer of *N*-glycans to protein or processing from high mannose to hybrid and complex structures (Pan and Elbein, 1985). Nonetheless, *N*-glycosylation is not required for GPI-anchoring or the correct apical sorting of gD-1-DAF in MDCK cells (Lisanti *et al.*, 1990a).

Mannosamine blocks the production of endogenous GPI-anchored proteins

To examine the generality of mannosamine action on GPI-anchored proteins, we determined its effect on the production of endogenous MDCK GPI-anchored proteins (Figure 8, left panel). Untransfected MDCK cells were metabolically-labeled overnight in the presence or absence of mannosamine. GPI-anchored proteins were detected by sensitivity to release by PI-PLC. Mannosamine treatment drastically reduced the intensity of three GPI-anchored proteins with apparent molecular weights of 180, 85 and 35 kd, identical to GPI-anchored proteins previously described in MDCK cells using cell surface labeling techniques (Lisanti *et al.*, 1988, 1990b; Wilson *et al.*, 1990). The effects of mannosamine were not due to non-specific inhibition of protein synthesis as the total amount of protein-associated radioactivity present in detergent extracts was not reduced by mannosamine (Figure 8, right panel).

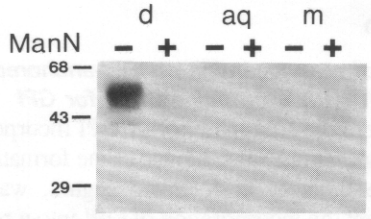

Fig. 6. Mannosamine treatment inhibits the incorporation of an anchor-specific component (ethanolamine). Plastic-grown cells were metabolically labeled overnight with [^{3}H]ethanolamine in the presence (+) or the absence (−) of mannosamine (ManN; 10 mM). Cell extracts were partitioned into detergent (d) and aqueous (aq) phases. Cell extracts and media (m) were then immunoprecipitated.

Mannosamine affects the synthesis of GPI precursors

The effect of mannosamine on GPI synthesis was evaluated in the well established system of *Trypanosoma brucei* (Menon *et al.*, 1988; Mayor *et al.*, 1990a,b; Figure 9), where many GPI structures have been characterized (Cross, 1990). Procyclic (insect stage) trypanosomes express an abundant GPI-anchored cell surface protein, procyclic acidic repetitive protein (PARP; Clayton and Mowatt, 1989), and synthesize a GPI-anchor precursor, PP1, which has recently been characterized as ethanolamine-P-Manα1-2 Manα1-6 Manα1-GlcN-(acylinositol)-P-1,-monoacylglycerol (Field *et al.*, 1991). Mannosamine was found to inhibit the incorporation of [^{3}H]ethanolamine into both PP1 and PARP, without affecting the synthesis of phosphatidyl-

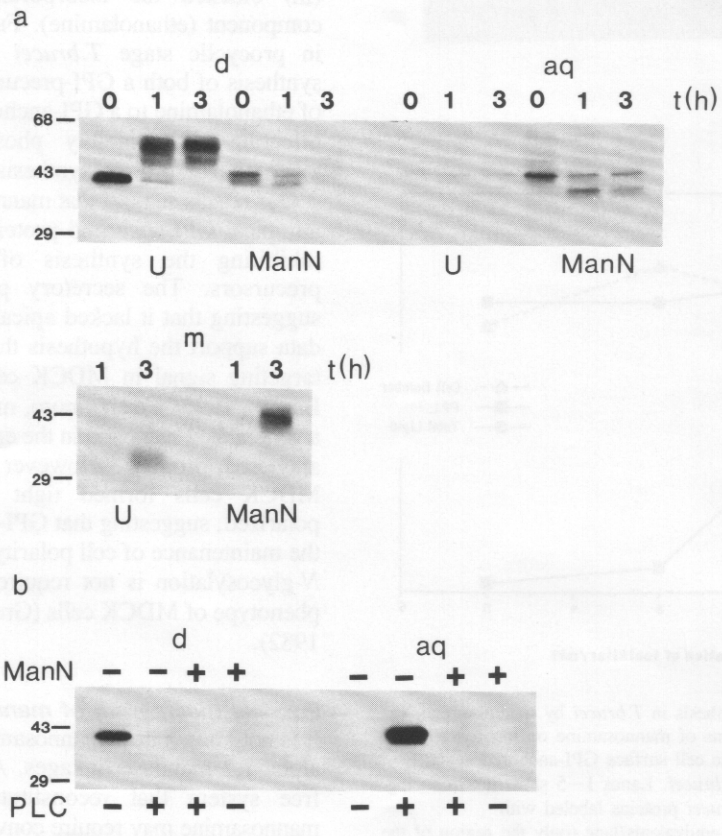

Fig. 7. Mannosamine induces a transient hydrophobic precursor of gD-1-DAF that is rapidly cleaved to a hydrophilic form and secreted. (**a**) Plastic-grown cells were pre-incubated for 4 h in glucose-free medium in the presence (ManN; 10mM) or absence (U) of mannosamine, pulsed for 20 min with ^{35}S-labeled amino acids and chased for various times (0, 1 and 3 h). Cell extracts (d, detergent; aq, aqueous) and media (m) samples were immunoprecipitated. (b) Plastic-grown cells were preincubated and pulsed for 20 min in the presence (+) or absence (−) of mannosamine (ManN; 10 mM) as above (0 h). Detergent phases were prepared and treated with PI-PLC (+ or −) as described in the legend of Figure 3 and Materials and methods. Detergent (d) and resulting aqueous (aq) phases were immunoprecipitated.

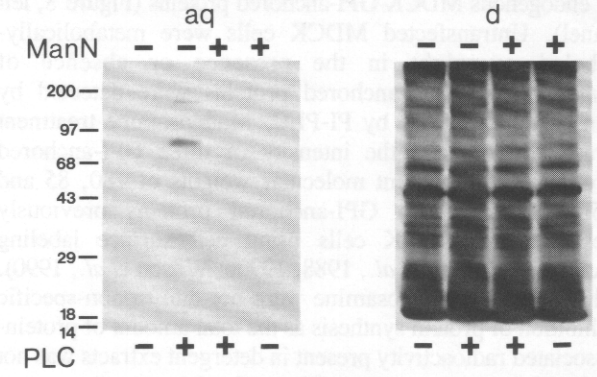

Fig. 8. Mannosamine treatment drastically reduces the detection of endogenous (PI-PLC sensitive) GPI-anchored proteins. Plastic-grown MDCK cells (untransfected) were pre-incubated for 3 h in glucose-free medium in the presence (+) or absence (−) of mannosamine (ManN; 10 mM) and metabolically labeled overnight with ^{35}S-labeled amino acids. Detergent phases were prepared from cell extracts and treated with PI-PLC (+ or −). Detergent (d; right panel) and resulting aqueous (aq; left panel) phases were precipitated with acetone and TCA, respectively and analyzed by SDS−PAGE and fluorography.

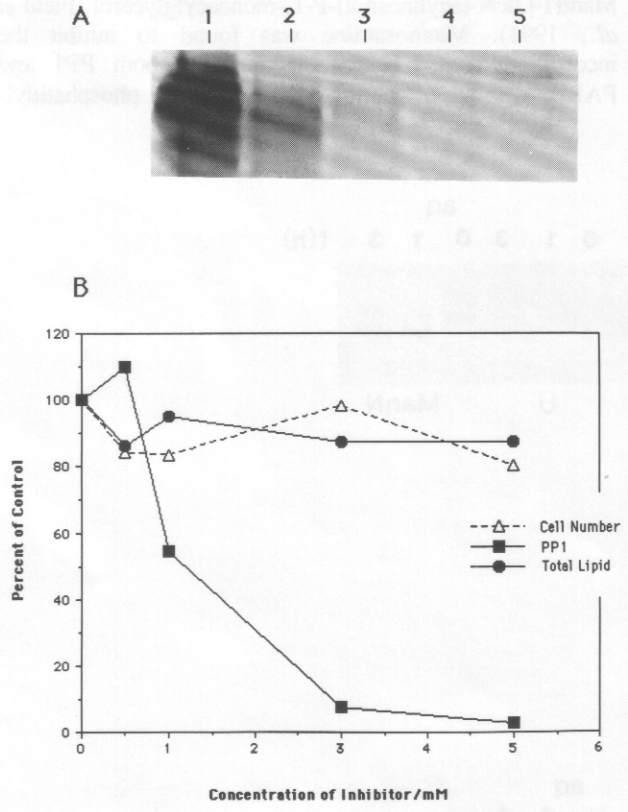

Fig. 9. Inhibition of GPI biosynthesis in *T.brucei* by mannosamine. (**a**) Effect of increasing concentrations of mannosamine on incorporation of [^{3}H]ethanolamine into PARP, the cell surface GPI-anchored acidic repetitive protein in procyclic *T.brucei*. Lanes 1−5 show non-reducing SDS−PAGE of delipidated *T.brucei* proteins labeled with [^{3}H]ethanolamine [1.6×10^7 cell equivalents/lane (only the region of the gel containing the PARP band is shown)]. Lane 1, untreated control; lanes 2−5, mannosamine treated (0.5, 1.0, 3.0 and 5.0 mM, respectively). (**b**) Effect of mannosamine on cell number and on the incorporation of [^{3}H]ethanolamine into PP1 (the major GPI in procyclic *T.brucei*; Field *et al.*, 1991) and total lipid (mostly phosphatidylethanolamine) following 16 h incubation with the inhibitor.

ethanolamine (PE) or compromising cell viability (Figure 9). Inhibition of the incorporation of [^{3}H]ethanolamine into both PP1 and PARP is consistent with the proposal that mannosamine exerts its effect by preventing the addition of the third mannose, an alpha 1,2 linked residue, to the core GPI glycan. Metabolic labeling with [^{3}H]mannosamine resulted in incorporation of ^{3}H into nucleotide sugar, lipid (with chromatographic properties similar to Dol-P-Man), lipids sensitive to tunicamycin, and into protein, suggesting that mannosamine can be incorporated into N-linked glycans, as well as GPI (not shown). Further experiments are underway to characterize in more detail the effect that mannosamine has on the biosynthesis of GPI in these organisms.

Discussion

The effect of mannosamine on GPI-anchored proteins is consistent with a trafficking role for GPI

We report here the first inhibitor of GPI incorporation into protein. Mannosamine, an inhibitor of the formation of alpha 1,2 mannose linkages in N-linked sugars, was found to radically affect the incorporation of GPI into a recombinant GPI-anchored protein transfected into the polarized epithelial cell line MDCK. Mannosamine treatment: (i) converted the major cell-associated form of the protein from a hydrophobic plasma membrane protein to a hydrophilic form that accumulated in the secretory pathway; (ii) led to the secretion of this hydrophilic protein in a non-polarized fashion; and (iii) blocked the incorporation of an anchor-specific component (ethanolamine). Furthermore, we showed that in procyclic stage *T.brucei* mannosamine prevents the synthesis of both a GPI-precursor lipid (PP1) and addition of ethanolamine to a GPI-anchored protein, PARP, without affecting cell viability phosphatidylethanolamine (PE) synthesis, or protein synthesis.

Our results suggest that mannosamine treatment converted an apical GPI-anchored protein to a secretory product by inhibiting the synthesis of transfer-competent GPI-precursors. The secretory product was non-polarized, suggesting that it lacked apical sorting information. These data support the hypothesis that GPI behaves as an apical targeting signal in MDCK cells (Lisanti and Rodriguez-Boulan, 1990). Furthermore, mannosamine treatment caused a generalized reduction in the expression of endogenous GPI-anchored proteins. However, the mannosamine treated MDCK cells formed tight monolayers and remained polarized, suggesting that GPI-anchoring is not required for the maintenance of cell polarity by MDCK cells. Similarly, *N*-glycosylation is not required to maintain the polarized phenotype of MDCK cells (Green *et al.*, 1981; Meiss *et al.*, 1982).

Possible mechanism of mannosamine action

It is not known how mannosamine inhibits the formation of alpha 1,2 mannose linkages. As it is uneffective in a cell-free system that reconstitutes N-linked glycosylation, mannosamine may require conversion to an active metabolite (Pan and Elbein, 1985). One possibility is that it is transferred to glycans from a mannose donor (GDP-Man and/or Dol-P-Man) and subsequent sugars cannot be added to the 2 position, since a primary amino group replaces the hydroxyl group at this position. Alternatively, it may act as

an enzymatic inhibitor of alpha-1,2-specific mannosyl transferases. Both scenarios would explain why mannosamine inhibits specifically the incorporation of ethanolamine into the *T.brucei* GPI-precursor lipid PP1, since ethanolamine is normally attached to a mannose residue of the conserved GPI-core structure that is alpha 1,2 linked. Our observations with trypanosomes favor the first hypothesis (a chain termination scheme), but the two mechanisms are not mutually exclusive. We are currently analyzing GPI-lipids and water soluble metabolites in cells labeled with [^{3}H]mannosamine to determine in detail the mechanism of action of the inhibitor.

GPI-anchored proteins are believed to be synthesized with a C-terminal hydrophobic extension that acts as a transient membrane anchor, prior to addition of GPI. This C-terminal peptide is removed and replaced with the GPI-anchor within 1−2 min of synthesis (Bangs *et al.*, 1985; Ferguson *et al.*, 1986; Conzelmann *et al.*, 1987). As this trans-peptidation (or trans-amidation) reaction occurs so rapidly, the detection of transient full length precursor proteins in live cells has been unsuccessful. In pulse−chase experiments with mannosamine, we detected a transient hydrophobic precursor that was rapidly degraded ($t_{1/2}$ = 1 h). This precursor was 1−3 kd larger than the precursor found in untreated control cells and was converted to a hydrophilic cell-associated form and secreted. Our results suggest that if transfer competent GPI-precursors are lacking, the 'GPI-transpeptidase' might express its proteolytic activity. This interpretation assumes that the enzyme responsible for proteolytic cleavage in mannosamine-treated cells is the same enzyme responsible for addition of the GPI-anchor in untreated cells. This possibility is supported by the observation that the mannosamine-induced secretory form is of identical molecular weight to an alternative form of the protein that lacks a C-terminal GPI-attachment signal. However, to be absolutely sure that cleavage occurs at the correct processing site requires knowledge of the structure of the C-terminal end of the mannosamine-induced secretory form. Similarly, the 'GPI-transpeptidase' can correctly proteolytically process the C-terminally uncleaved translation product of a GPI-anchored protein *in vitro*, without adding a GPI moiety (Bailey *et al.*, 1989).

Mannosamine treatment mimics the phenotype of anchor-deficient states

Cell lines expressing a single anchor-minus form of a GPI-anchored protein have been derived by transfection with deletion mutant cDNAs lacking a complete C-terminal GPI-attachment signal (Caras *et al.*, 1989; Powell *et al.*, 1991). However, several cell lines exist that express a generalized form of an anchoring defect and do not display cell surface forms of GPI-anchored proteins. L cells secrete both endogenous and transfected GPI-anchored proteins (Ly-6, Qa, N-CAM 120), without expressing a hydrophobic or cell surface membrane-associated form (Stroynowski *et al.*, 1987; Singh *et al.*, 1988). A well-studied case is that of Thy-1 negative mutant lymphoma cells (selected with anti-Thy-1 antibodies and complement), which are defective in cell surface transport of Thy-1 (a GPI-anchored protein) and do not express endogenous (PI-PLC sensitive) GPI-anchored proteins (Conzelman *et al.*, 1986, 1988; Fatemi and Tartakoff, 1986, 1988). Using the same cell line, different groups either detected a hydrophobic precursor (PI-PLC resistant) or found that a hydrophilic form of Thy-1 was secreted to the culture medium. This secreted form did not incorporate ethanolamine (an anchor-specific component; Fatemi and Tartakoff, 1988). In one case (complementation group E), the defect that leads to the anchor-minus state appears to be in the synthesis of Dol-P-Man and transfection with the yeast Dol-P-Man synthetase gene restores surface expression of GPI-anchored proteins (Thy-1 and Ly-6; DeGasperi *et al.*, 1990). This is in agreement with the role of Dol-P-Man as donor for all three of the mannose residues in the conserved core of the GPI-glycan (Menon *et al.*, 1990a). These observations indicate that a defect in mannose addition can block the addition of the GPI-anchor, resulting in generalized loss of surface expression and secretion of GPI-anchored proteins, a very similar phenotype to the one we observed in mannosamine-treated MDCK cells.

Certain human diseases may also present the anchor-deficient phenotype. Paroxysmal nocturnal hemoglobinuria is characterized by the absence, or low-level expression, of cell surface forms of GPI-anchored proteins (e.g. alkaline phosphatase, DAF, AChE, FcR III, C8 binding protein and homologous restriction factor) (Low and Saltiel, 1988; Ferguson and Williams, 1988; Low, 1989). Another pathological state that may lead to an anchor-deficient phenotype is diabetes. Interestingly, in diabetic states the serum levels of several GPI-anchored proteins are elevated (Karnieli *et al.*, 1987; Skillen *et al.*, 1987) and the levels of Dol-P-Man are reduced (Scharma *et al.*, 1987). This may be due to glucose starvation which has been shown to result in the formation of truncated N-linked glycans, identical to those of mutant cells defective in Dol-P-Man synthesis, presumably by inhibition of Dol-P-Man transfer (Chapman and Calhoun, 1988). As Dol-P-Man is required for the synthesis of transfer competent GPI-precursors (Menon *et al.*, 1990a), a diabetic anchor-deficient phenotype might be explained by depletion of GPI-precursor lipids induced by glucose starvation.

In summary, mannosamine treatment biochemically induces a phenotype previously observed only as a consequence of deletion of the C-terminal signal for GPI-attachment or a genetic defect in GPI synthesis.

Materials and methods

Reagents

Protease inhibitors and amino sugars were from Sigma. Recombinant phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was the generous gift of Martin Low (Columbia University, NY). Sources for anti-gD-1 rabbit polyclonal (for immunoprecipitation) and mouse monoclonal antibodies (for immunofluorescence) were as described (Lisanti *et al.*, 1989). Biotinylating reagents were from Pierce.

Constructs

gD-1-DAF, a GPI-anchored fusion protein, contains 300 of the 340 amino acids encoding the ectodomain of gD-1 (a type 1 transmembrane protein) fused to the 37 C-terminal amino acids of DAF that constitute its GPI-attachment signal. Truncated gD-1 is identical to gD-1-DAF, except it does not contain a signal for GPI-attachment. Both constructs were described elsewhere (Caras *et al.*, 1987).

Cell culture

Transfected MDCK (type II) cells expressing GPI-anchored (gD-1-DAF) or secreted (truncated gD-1) forms of gD-1 were derived and characterized as described previously (Lisanti *et al.*, 1989). Cells were maintained in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), non-essential amino acids, and antibiotics (normal medium). For experiments, cells were seeded at high density in 35 mm dishes (plastic-grown) or on Transwells (24.5 mm, 0.4 μ pore, tissue-culture treated polycarbonate filters; filter grown) and used on the second or the fourth day after plating, respectively. Media (as above) containing

sodium butyrate (10 mM) was added 12–16 h before use to elevate the expression of the transfected gene products (Gorman and Howard, 1983; Gottlieb *et al.*, 1986). Untransfected MDCK cells were cultured as described above, except sodium butyrate treatment was omitted.

Metabolic-labeling

For steady-state labeling, cells were incubated overnight (16–20 h) with 100 μCi/ml Expre^{35}S^{35}S-label (NEN) in HEPES-buffered (pH 7.3) MEM containing one-tenth the normal concentration of methionine (Met) and cysteine (Cys), 0.2% BSA, 1% FBS and 10 mM sodium butyrate (Lisanti *et al.*, 1989). Similarly, for pulse–chase experiments cells were starved (10 min) in MEM lacking Met and Cys, and pulsed for 20 min with 1 mCi/ml Expre^{35}S^{35}S-label (NEN) and chased for various times in medium containing excess Met and Cys. Steady-state labeling with 1-[^{3}H]ethanolamine (NEN; 100 μCi/ml) was performed in normal medium described above. Metabolically labeled proteins were visualized after SDS–PAGE (10%) by fluorography with sodium salicylate (Chamberlain, 1979).

Biotin polarity assay

Filter-grown cells were biotinylated at 4°C by addition of sulfo-NHS-biotin to the upper (apical) or the lower (basolateral) compartment of the filter chamber, respectively (Lisanti *et al.*, 1988; Sargiacomo *et al.*, 1989). Alternatively, to monitor the kinetics of cell surface delivery, cell surface amino groups were quenched and cells were recultured at 37°C for various times prior to biotinylation (Lisanti *et al.*, 1990a). Biotinylated proteins were visualized after transfer to nitrocellulose by blotting with [^{125}I]streptavidin and autoradiography (Sargiacomo *et al.*, 1989).

Treatment with protease inhibitors

After cell-surface biotinylation or steady-state metabolic labeling, cells were recultured for 8 h in DMEM containing 0.2% BSA and antibiotics in the absence or presence of different protease inhibitors. Leupeptin, pepstatin, antipain, and chymostatin (10 mg/ml stocks prepared with DMSO) were used at 100 μg/ml; aprotinin (bovine pancreatic trypsin inhibitor) and soybean trypsin inhibitor (10 mg/ml stocks prepared with 20 mM HEPES, pH 7.3) were used at 500 μg/ml; E-64 (a 25 mg/ml stock prepared with deionized water and ethanol, 1:1) was used at 500 μg/ml; and chloroquine (a 10 mg/ml stock prepared with deionized water) was used at 100 μg/ml. Aliquots were stored at −20°C and diluted into the appropriate media immediately before use. Control experiments performed with solvents alone showed no difference from untreated samples. Similar concentrations were used by others to inhibit proteolytic activites (Tanaka *et al.*, 1986; Musil and Baenziger, 1987; Urban *et al.*, 1987).

Mannosamine (2-amino-2-deoxy D-mannose) treatment

Cells were pre-incubated for various times (1–4 h) in glucose-free medium (RPMI 1640) containing 10% FBS and 10 mM sodium butyrate, in the absence or presence of mannosamine (10 mM). These concentrations and similar preincubation conditions were shown to produce optimal effects on N-linked glycan synthesis in MDCK cells (Pan and Elbein, 1985). For steady-state labeling experiments, the preincubation step was usually omitted and mannosamine was added only to the labeling media. During pulse–chase experiments, mannosamine was included during all the manipulations. A 1 M sterile-filtered stock of mannosamine was prepared with deionized water and frozen at −20°C. Aliquots were thawed and diluted into the appropriate media immediately before use.

Identical conditions were used to evaluate the effect of glucosamine on the biosynthesis of GPI-anchored proteins.

It should be noted that mannosamine was ineffective when added only from the basolateral compartment (not shown). As glucose prevents mannosamine uptake (Pan and Elbein, 1985), these results suggest that mannosamine may be taken up by the apical Na/glucose co-transporter.

PI-PLC assay

PI-PLC mediated cleavage of the GPI-anchor (i.e. loss of the diacylglycerol moiety) was monitored as a transition from a hydrophobic to a hydrophilic state, as described (Conzelmann *et al.*, 1986; Lisanti *et al.*, 1988, 1990b). Briefly, cell extracts were prepared with the detergent Triton X-114 and subjected to temperature-induced phase separation. Detergent phases were collected and treated with PI-PLC. The action of PI-PLC was evident by subsequent partitioning into the aqueous phase. Both detergent and aqueous phases were subjected to immunoprecipitation. All solutions contained inhibitors to the four distinct classes of proteases: leupeptin and antipain (serine and cysteine protease inhibitors); pepstatin (aspartyl protease inhibitor); EDTA (metalloprotease inhibitor).

Cell extraction and immunoprecipitation

Cells were extracted for 1 h on ice with 1 ml of Tris-buffered saline (TBS, 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA) containing 1% Triton X-114 and protease inhibitors (lysis buffer). Detergent and aqueous phases were prepared by temperature-induced phase separation, readjusted to 1% Triton X-114 and immunoprecipitated with a 1:150 dilution of rabbit anti-gD-1 IgG, as described (Lisanti *et al.*, 1989).

To determine if a fraction of gD-1-DAF remained insoluble, pellets formed after extraction with Triton X-114 were solubilized in 10 μl of 10% SDS by continuous vortexing. After solubilization, samples were diluted to 1 ml with lysis buffer for a final concentration of 0.1% SDS. Both soluble and insoluble fractions were then subjected to immunoprecipitation in parallel. Similar extractions were performed with 60 mM octyl glucoside (dissolved in TBS), as this concentration of octyl glucoside permits optimal solubilization of GPI-anchored proteins (Hooper and Turner, 1988). Despite reports that GPI-anchored proteins may be resistant to solublization, under our conditions both Triton X-114 (1%) and octyl glucoside (60 mM) effectively solubilized gD-1-DAF [>95% of both apical and basolateral fractions of the 45–50 kd form, as well as the 40 kd precursor form (not shown)]. The presence of 1% DMSO in the extraction mixture (from addition of protease inhibitors dissolved in DMSO) may explain why extraction with Triton X-114 was as effective as with octyl glucoside.

Media samples (1 ml) were microfuged for 30 s to remove cellular debris, protease inhibitors added, and adjusted to 1% Triton X-114. After 1 h on ice, media samples were phase separated, the aqueous phases were collected and readjusted to 1% Triton X-114 prior to immunoprecipitation.

Immunofluorescence

Cells were seeded at high density on glass coverslips (1–2 days confluent) and incubated 12–16 h in media containing sodium butyrate. Monolayers were fixed with 2% paraformaldehyde, permeabilized with saponin (Reggio *et al.*, 1983; Rodriguez-Boulan, 1983), and incubated with a 1:100 dilution of mouse anti-gD-1 monoclonal IgG_{2a}. Bound antibodies were visualized with the appropriate rhodamine-conjugated secondary antibody.

Culture and metabolic labeling of trypanosomes

Procyclic stage *Trypanosoma brucei* 427 were cultured as described (Field *et al.*, 1991) and metabolically-labeled with [1-^{3}H]ethanolamine (Amersham Corp.) at 20 μCi/ml or with [6-^{3}H]-D-mannosamine (American Radiolabeled Chemical Inc.) at 40 μCi/ml in sugar-free RPMI-1640 medium at a density of 2×10^7 parasites/ml. For ethanolamine labeling, mannosamine (0–5 mM) was added to the cultures 1 h before addition of radiolabel. Mannosamine labeling was carried out in the absence or presence of tunicamycin (2 μg/ml). Cells were harvested 16 h later, and lipids were extracted with chloroform:methanol:water (10:10:3, vol:vol:vol). Cell number following incubation overnight with the inhibitor was determined by counting the cells in a hemocytometer. Total incorporation of radiolabel into lipid was estimated by liquid scintillation counting of an aliquot of the organic extract. The lipid extracts were analyzed by thin layer chromatography as described (Field *et al.*, 1991). The delipidated cell pellet was solubilized in 50 μl of 5% SDS (overnight at room temperature), and an aliquot analyzed by 15% non-reducing SDS–PAGE.

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