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GLYCOPINION

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Glycoproteins from a diversity of eukaryotic organisms have glycosylphosphatidylinositol (GPI) membrane anchors. GPI-anchored proteins have been identified in organisms as diverse as fungi (*S. cerevisiae*), molluscs (squids), trypanosomatids and man.

Until recently, little was known about their biosynthesis or function, or indeed why some proteins should use a GPI anchor rather than a classical hydrophobic transmembrane peptide. Many roles have been proposed for glycan anchors including the shedding and turnover of membrane proteins, signal transduction and intracellular targeting. In the case of *Trypanosoma brucei* variant surface glycoprotein (VSG) it is possible that the glycan has a space filling role in the VSG coat, while in rat thymocyte Thy-1 preliminary data suggest that the glycan part of the anchor may fit into a pocket in the protein allowing extensive direct interactions between the Thy-1 peptide and the membrane surface. The chemical characterization of a number of glycan anchors, including those attached to *T. brucei* VSG, to rat brain Thy-1 and to bovine erythrocyte acetylcholinesterase, has revealed that they all contain a consensus core sequence although there is diversity in both the glycan structures and the glycerol linked aliphatic substituents.

The attachment of a GPI anchor represents a novel pathway for the covalent addition of a new class of glycan to a polypeptide chain: it is this pathway and, in particular, the role of inositol acylation which is discussed by Dr Mark Field of the Rockefeller University.

Some readers may find a general review [e.g. 1–3] a useful introduction to this article which focuses on one particular aspect of GPI anchor biosynthesis.

Some questions which it raises include:

- Is there a rationale for the use of a GPI anchor rather than a classical hydrophobic transmembrane peptide?
- Do functional differences result from alternatively anchored forms of the same protein?
- What is the function of inositol acylation in the biosynthesis of glycolipids and is this process under developmental control?
- Inositol acylation renders GPI anchors resistant to PI specific phospholipase C. Where are the processing enzymes located which convert inositol acylated GPI's to the phosphatidylinositol phospholipase C (PI-PLC) sensitive form?

Inositol acylation of glycosylphosphatidylinositol membrane anchors: what it is, and why it may be important

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A large number of membrane proteins are associated with the lipid bilayer via post-translational attachment of a complex inositol-containing glycolipid (GPI). The GPI is the sole mechanism of membrane localization amongst the proteins containing this modification (see [4–5] for lists of GPI-anchored proteins). This has attracted much interest, especially amongst glycobiologists, as attachment of a GPI anchor represents a novel pathway for the covalent addition of a new class of glycan to a polypeptide chain. Whilst there is no clear cut rationale for the use of a GPI anchor for membrane association rather than a classical hydrophobic transmembrane peptide, some functions are emerging for this class of membrane proteins. For example, GPI-anchored proteins are preferentially targeted toward the apical surface of polarized epithelia [6], and some GPI-anchored proteins are clearly involved in T-cell activation [7] or can act as cytokine receptors [8]. In this article data are presented to support an hypothesis for a GPI anchor processing pathway, which deals primarily with the lipid portion of the GPI moiety (Fig. 1).

A large body of data, accrued over the past five years, has provided a detailed picture of the biosynthesis of the GPI

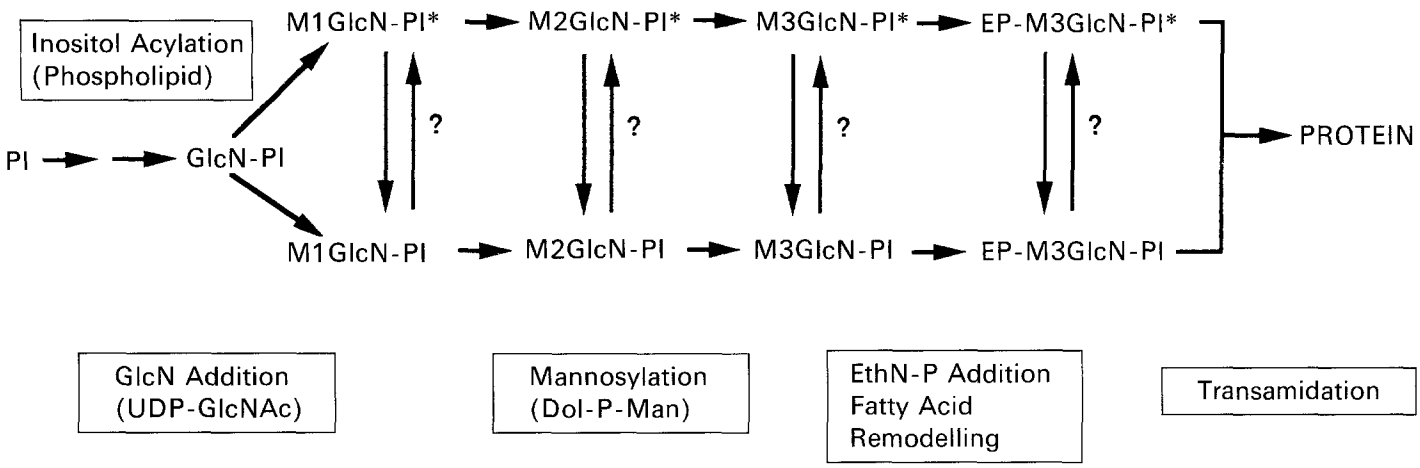

Figure 1. Biosynthesis of GPI anchor precursor lipids. The scheme is based on data from several laboratories, and has been simplified for clarity. The processes taking place at different points in the pathway are boxed. The upper arm of the pathway is the inositol-acylated GPI lipids, whilst the lower is the non-inositol-acylated. In mammalian cells and in procyclic form of *T. brucei* the upper arm pathway predominates. In mammalian bloodstream form *T. brucei* the lower pathway is predominant, except for the final stages when both inositol-acylated and non-inositol-acylated forms of the EP-M3GlcNPI lipids (P2 and P3 respectively) are present in high levels. The relationship between the two pathways is not well understood, i.e. no data are available on possible interconversion of the acyl/nonacyl forms of the same lipid, or if the metabolic flux through one arm exceeds that through the other. It is quite likely that such details will be different in different organisms/cells. It is also important to note that it is not known whether one or both of the EP-containing lipids can act as a GPI-anchor donor *in vivo*. Abbreviations used are: PI, phosphatidylinositol; GlcN, glucosamine; M(1-3), mannose residues (1-3); EP, ethanolamine phosphate; PI*, inositol-acylated PI.

anchor and processing of the nascent polypeptide. The paradigm system for these studies has been the bloodstream form of the African trypanosome, *T. brucei*. The surface of these organisms is covered with a dense coat of a single glycoprotein, the variant surface glycoprotein (VSG) (ca 1×10^7 copies per cell, accounting for 10% of total cell protein synthetic output). The VSG is anchored to the cell membrane by a GPI anchor attached to the α-carboxyl group of the C-terminal amino acid via an ethanolamine–phosphate bridge. Since the structure of the GPI from VSG was elucidated [9] several other GPI anchors have been characterized. All contain the consensus core sequence R–EthN–P–Man–Man–Man–GlcN–PI (where R represents the glycoprotein, and PI is phosphatidylinositol). The presence of various additional substituents on the core has been documented, e.g. α-galactose [9], ethanolamine-phosphate (in Thy-1 and acetylcholinesterase [10–12]), additional mannose residues (Thy-1, *S. cerevisiae* GAS1 gene product (gp125) and the *T. cruzi* 1G7 antigen [3]) and GalNAc (Thy-1). Presumably the addition of mono-saccharides to the GPI core takes place in the Golgi stacks, along with processing of N and O-linked glycans. The PI moiety can vary in different anchors, for example the VSG PI contains exclusively dimyristylglycerol [13], whereas in the GPI from human erythrocyte acetyl-cholinesterase (E^{hu}AchE), the PI is an alkylacylglycerol structure [11–12]. Interestingly, in the case of VSG, the PI moiety does not begin as dimyristylglycerol; this structure is the result of specific fatty acid 'remodelling' [14]. In the case of other GPI anchors, it appears that the PI selected for glycosylation is a small subpopulation of the total cellular PI pool [15–16].

The GPI anchor is added very rapidly after synthesis of the polypeptide. A hydrophobic C-terminal GPI signal peptide, important in directing the addition of the GPI, is removed and replaced by a preformed GPI precursor. In *T. brucei* bloodstream form, two glycolipid precursors have been identified (P2 and P3 [17]) both of which are essentially identical to the VSG GPI anchor itself, with the exception that one of them, P3, contains an extra fatty acid (palmitate) directly esterified to the inositol ring [18]. The fatty acid is probably derived from a phospholipid donor [16]. This modification renders P3 resistant to cleavage by PI-specific phospholipase C (PI-PLC). The VSG GPI anchor itself is completely sensitive to the PI-PLC, so that for a time it was thought that P3 was an interesting side product of the system, and not necessarily important in the main pathway.

However, some mature glycoproteins contain GPI anchors with essentially P3 type structures, i.e. inositol acylated. For example, E^{hu}AchE [11–12], and the *T. brucei* procyclic stage (insect) specific antigen procyclic acidic repetitive protein (PARP) [16, 19]. Therefore in some proteins, inositol acylation is part of a mature structure. The observation that the insect stage of *T. brucei* synthesized inositol acylated GPI anchors, and not PI-PLC sensitive structures, suggests that inositol acylation is under developmental control. Analysis of the GPI precursors in the procyclic stage trypanosome identified only inositol-

acylated precursors, i.e. GPIs more similar to P3 than to the VSG GPI anchor homologue P2 [20]. It is important to note that P3, P2 and the PARP GPI-precursor (PPI) can all be added to the VSG polypeptide using an *in vitro* system [21], so that the absence of the P3 type GPI anchor from mature VSG is unlikely to be due simply to the inability of the bloodstream transfer machinery to use P3 as a GPI-precursor. Further studies of the procyclic GPI anchor biosynthetic pathway have shown that the level of inositol-acylation of the earlier intermediates is much greater compared to the bloodstream form [22], but that interestingly the first glycosyl-inositol intermediate, GlcN–PI, is not inositol-acylated. Only after the formation of a Man–GlcN–PI is the presence of the additional fatty acid detected.

These data suggest that an enzymatic activity present in bloodstream form trypanosomes is capable of removing the acyl-group from the inositol of the GPI anchor or precursor, and that this enzyme is down-regulated in the procyclic form. An alternative explanation would be that the addition of acyl groups to the inositol is more efficient in the procyclic form. Evidence supporting the former hypothesis came from analysis of murine L-cell lines, where it was shown that resistance of GPI-anchored proteins to cleavage by PI-PLC (i.e. presence of an acyl moiety on the GPI inositol) behaved as a recessive trait in cell fusion experiments [23], consistent with the presence of an inositol-specific acylhydrolase in the cells with PI-PLC sensitive GPI-anchored proteins.

These observations suggest that an inositol-specific acylhydrolase is present in some cells which converts GPI-anchored proteins into a PI-PLC sensitive form. In cells where this activity is absent, GPI-anchored proteins are not PI-PLC sensitive, e.g. procyclic *T. brucei*, human erythrocytes. This model still, however, leaves unanswered the question of the role of the inositol-acylated GPI-anchor precursor, P3, in VSG biosynthesis. No evidence has been obtained about the processing of this species, i.e. whether P3 is converted to P2, or alternatively added to VSG, and then de-acylated. At present there is no direct evidence to support either pathway, but a number of observations have been made which are consistent with the latter model, and these are outlined below.

In T cells, the best characterized GPI-anchored protein is Thy-1 [10]. Thy-1 is efficiently released from the cell surface by PI-PLC, suggesting that most, if not all, of the Thy-1 molecule's GPI anchors are sensitive to the lipase. Interestingly, recent work has shown that the probable GPI anchor precursors in these cells are PI-PLC resistant [24], suggesting that conversion of the GPI moiety from a PI-PLC resistant to a sensitive form takes place after synthesis of the completed precursor, which could be following attachment of the glycolipid to the C-terminus of the polypeptide. Alternatively, rapid processing of the precursor could take place just prior to addition to the polypeptide. Though the structural basis for the resistance of the mammalian GPI-precursors to PI-PLC has not been reported, it is most likely that this is due to acylation of the inositol residue. In this regard it is important to note that the position of the palmitate in the inositol has not been directly determined, so that the precise relationship between inositol palmitylation and PI-PLC resistance is not fully characterized.

A species with the properties of an acyl-glucosamine-PI has been identified in both a T-cell hybridoma and a dolicholphosphomannose synthase deficient yeast mutant [25–26]. The presence of these GPI species increases the likelihood that acylation occurs at an early stage in the glycosylation of PI, with de-acylation being a late maturation step. Note that in the analysis of trypanosome GPI biosynthesis *in vitro*, acyl-inositol structures are not seen this early in the pathway [22, 27]. However, these observations should be interpreted with caution as they have been made with mutant cells or cell-free systems, and may not faithfully reflect the true physiological situation.

Further data on AchE processing is consistent with processing following addition. In different sublines of human erythroleukemia K562 cells, mature AchE is either PI-PLC sensitive or resistant [28], i.e. a mixed population is not seen. Most significant is the observation that, regardless of the PI-PLC sensitivity of the mature AchE, molecules which are insensitive to sialidase are also resistant to PI-PLC, suggesting that AchE does not become PI-PLC sensitive until it reaches a compartment where sialylation takes place, i.e. the *trans*-Golgi.

When bloodstream form trypanosomes are lysed under mild conditions in the presence of a detergent the VSG is completely released as a hydrophilic protein, due to removal of diacylglycerol by an endogenous PI-PLC [13]. In pulse-chase studies, VSG is apparently sensitive to this enzyme within approximately one minute of synthesis (as detected by appearance of the cross-reactive determinant [29]. Lysis by hypotonic shock, which is not expected to completely rupture internal membrane bounded organelles, resulted in very little release of VSG after 10 minutes of a chase, but in near quantitative release following 40 minutes chase [29]. In addition, if the cells are lysed in boiling sodium dodecylsulphate (to denature all proteins), VSG takes a full 15 minutes to become PI-PLC sensitive [30]. A highly probable explanation for these observations is that the VSG GPI-anchor is in fact inositol-acylated, and reaches a compartment where it is processed at 15 minutes. This timing is important, as this is close to the expected transit time for a glycoprotein to reach the Golgi stack from the ER, and therefore is consistent with the data of Toutant *et al.* [28] for AchE. The reason for complete release of VSG following native detergent lysis could be due to solubilization of a microsome-contained inositol-specific acyl-hydrolase. This is evidence for a transiently PI-PLC resistant anchor structure in VSG.

The evidence presented above for an inositol-specific

acylhydrolase suggests the following model for GPI-anchor processing. In most cell types GPI anchor precursors are synthesized as PI-PLC resistant molecules which are added to the nascent polypeptide as it emerges into the ER lumen. At some point during subsequent transit of the protein to the cell surface, the GPI anchor inositol is de-acylated, which converts the protein to its mature PI-PLC sensitive form. In some cell types, this processing enzyme has been lost (e.g. human erythrocytes and K562 cells, some murine L-cells), or is developmentally down regulated (procyclic form *T. brucei*). There are, of course, exceptions, e.g. in *Leishmania* species, no acyl-inositol GPI structures have been detected, either as lipids or as proteins.

Complicating this picture is the occurrence of P2 in the bloodstream form trypanosome, and the earlier PI-PLC sensitive GPI anchor biosynthetic intermediates in this organism [27]. It is probable that the GPI lipids are also able to move to post-ER compartments, e.g. in bloodstream form trypanosomes GPI lipids containing α-galactose have been detected [31], and similar observations have been made in the procyclic form [20]. This may account for the presence of P2 in the trypanosome extracts. One piece of data contradictory to this model is that an apparent complete inhibition of P3 biosynthesis by treatment of living trypanosomes with phenyl methane sulphonyl fluoride (PMSF) does not affect VSG anchoring [32]. This could imply that P3 has nothing to do with VSG anchoring *per se*, but 90% inhibition of both P2 and P3 synthesis by fluoroglucose also does not alter VSG biosynthesis [33], suggesting that GPI precursors are synthesized in such vast excess that even a small amount of residual synthesis is sufficient for normal VSG-anchoring. Alternatively, de-acylation of P3 and VSG inositol could be performed by two different enzymes, located in the ER and the Golgi, so that P3 can be de-acylated in a more proximal compartment than VSG, explaining the presence of the PI-PLC sensitive structures in bloodstream trypanosomes.

What, then, is the function of inositol acylation? It has been suggested that acylation of the inositol is important in altering the conformation of the PI headgroup and allowing selection of a subpopulation of PI molecules for glycosylation [34]. 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 [22], so that mannosylation may be the reaction affected, i.e. acylation of GlcN–PI could be a signal for the further elaboration of the glycolipid, and may be a kinetic synthesis control point. A further possibility is that the modification of the inositol is important in translocation of the nascent GPI precursor from the cytoplasmic face of the ER (where PI is synthesized) to the lumenal face, where it is added to protein. However, this cannot be the whole story, as *Leishmania* do not make acyl–inositol GPI structures, and the mature GPI anchor precursor is PI-PLC sensitive [35]. Further data regarding the enzyme specificity and the topology of GPI biosynthesis will be required to assess the potential role of inositol-palmitylation.

In this article, I have argued that in most instances GPI-anchors are added to nascent polypeptides as PI-PLC resistant structures, due to the presence of an acyl group esterified to the inositol ring. The GPI is then converted to a PI-PLC sensitive form by the action of an inositol-specific acylhydrolase, which is located in a post-ER compartment, possibly the Golgi apparatus. In some cells the acylhydrolase is inactive, and may be under developmental control, e.g. stage-specific expression in the African trypanosome, and lineage specific in erythropoiesis. Certain predictions can be made from this model. Firstly, the bloodstream form trypanosome is expected to contain an inositol-specific acylhydrolase, whilst the procyclic is not, and secondly, for a short time following biosynthesis, GPI-anchored proteins should be resistant to cleavage by PI-PLC. Both of these predictions are eminently testable, and if proved correct will demonstrate further complexity in the post-translational modification of glycoproteins.

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References

1. Ferguson MAJ, Williams AF (1988) *Ann Rev Biochem* **57**:285–320.
2. Doering TL, Masterson WJ, Englund PT, Hart GW (1990) *J Biol Chem* **265**:611–4.
3. Ferguson MAJ (1991) *Curr Opin Struct Biol* **1**:522–9.
4. Low MG (1989) *Biochim Biophys Acta* **988**:427–54.
5. Field MC, Menon AK (1992) *CRC Handbook: Lipid Modifications of Proteins* (Schlessinger, ed) In press.
6. Lisanti MP, Rodriguez-Boulan E (1990) *Trends Biochem Sci* **15**:113–8.
7. Robinson PJ (1991) *Immunol Today* **12**:35–41.
8. Davis S, Aldrich TH, Valenzuela DM, Wong V, Furth ME, Squinto SP, Yancopoulos GD (1991) *Science* **253**:59–63.
9. Ferguson MAJ, Homans SW, Dwek RA, Rademacher TW (1988) *Science* **239**:753–9.
10. Homans SW, Ferguson MAJ, Dwek RA, Rademacher TW, Anand R, Williams AF (1988) *Nature* **333**:269–72.
11. Roberts WL, Myher JJ, Kuksis A, Low MG, Rosenberry T (1988) *J Biol Chem* **263**:18766–75.
12. Roberts WL, Santikarn S, Reinhold V, Rosenberry T (1988) *J Biol Chem* **263**:19776–8784.
13. Ferguson MAJ, Haldar K, Cross GAM (1985) *J Biol Chem* **260**:4963–8.
14. Masterson WJ, Raper J, Doering TL, Hart GW, Englund PT (1990) *Cell* **62**:73–80.

15. Butikofer P, Kuypers FA, Shackleton C, Brodbeck U, Stieger S (1990) *J Biol Chem* **265**:18983–7.
16. Field MC, Menon AK, Cross GAM (1991) *EMBO J* **10**:2731–9.
17. Menon AK, Mayor S, Ferguson MAJ, Duszenko M, Cross GAM (1988) *J Biol Chem* **263**:1970–7.
18. Mayor S, Menon AK, Cross GAM (1990) *J Biol Chem* **265**:6174–81.
19. Clayton C, Mowatt M (1989) *J Biol Chem* **264**:15088–93.
20. Field MC, Menon AK, Cross GAM (1991) *J Biol Chem* **266**:8392–400.
21. Mayor S, Menon AK, Cross GAM (1991) *J Cell Biol* **114**:61–71.
22. Field MC, Menon AK, Cross GAM (1992) *J Biol Chem* **267**:5324–9.
23. Singh N, Singleton D, Tartakoff AM (1991) *Molec Cell Biol* **11**:2362–74.
24. DeGasperi R, Thomas LJ, Sugiyama E, Chang HM, Beck PJ, Orlean P, Albright C, Waneck G, Sambrook JF, Warren C, Yeh ET (1990) *Science* **250**:988–91.
25. Orlean P (1990) *Mol Cell Biol* **10**:5796–805.
26. Sugiyama E, DeGaspari R, Urakaze M, Chang H-C, Thomas LJ, Hyman R, Warren CD, Yeh ETH (1991) *J Biol Chem* **266**:12119–22.
27. Menon AK, Schwarz RT, Mayor S, Cross GAM (1990) *J Biol Chem* **265**:9033–44.
28. Toutant JP, Richards MK, Krall JA, Rosenberry T (1990) *Eur J Biochem* **187**:31–8.
29. Ferguson MAJ, Duszenko M, Lamont GS, Overath P, Cross GAM (1986) *J Biol Chem* **261**:356–62.
30. Bangs JD, Andrews NW, Hart GW, Englund PT (1986) *J Cell Biol* **103**:255–63.
31. Mayor S, Menon AK, Cross GAM (1992) *J Biol Chem* **267**:754–61.
32. Masterson W, Ferguson MAJ (1991) *EMBO J* **10**:2041–5.
33. Schwarz RT, Mayor S, Menon AK, Cross GAM (1989) *Biochem Soc Trans* **17**:746–8.
34. Doering TL, Masterson WJ, Englund PT, Hart G (1989) *J Biol Chem* **264**:11168–73.
35. Field MC, Medina-Acosta E, Cross GAM (1991) *Mol Biochem Parasitol* **48**:277–30.

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