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Sidedness of Biosynthesis of Glycosylphosphatidylinositol Anchors in the Endoplasmic Reticulum of *Saccharomyces cerevisiae*

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Abstract. Many surface membrane glycoproteins of eucaryotes are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor. Biosynthesis of these anchors proceeds through two stages. First, the synthesis of the protein and of a free glycosylphosphatidylinositol (GPI) is achieved separately. In a second step, the protein is hooked onto the preformed free GPI whereby a provisional C-terminal hydrophobic peptide is removed. The GPI-anchored protein is subsequently transported to the cell surface by way of vesicular traffic. It is presumed that the attachment of the preformed free GPI's to proteins occurs on the luminal surface of the endoplasmic reticulum (ER). The stepwise addition of sugars by glycosyltransferases onto phosphatidylinositol to form a free GPI is equally presumed to occur in the ER, but it is unclear whether these reactions take place at the cytosolic or the luminal side of the membrane. Here we tried to get some information on the membrane orientation of free GPIs in *Saccharomyces cerevisiae* surmising that their orientation might tell us something about the probable location of the biosynthetic process. When using trinitrobenzenesulfonic acid as a probe, we find that 75% of the free GPIs in intact ER-derived microsomes get derivatized, whereas 100% get derivatized in detergent-permeabilized microsomes. This finding is compatible with the idea that in yeast lipid anchors are built up at the cytosolic surface.

Introduction

Many proteins need to be fixed to an intracellular or surface membrane in order to function properly. Most membrane proteins are anchored to the membrane by way of membrane-spanning hydrophobic sequences. However, recently many kinds of co- or posttranslational protein modification through covalent attachment of lipids to membranes have been found, and in most cases the lipid attachment results in a stable association of proteins with membranes, since the bound lipids will seek the hydrophobic environment of membranes [1][2]. The discoveries of myristic acid, farnesyl or geranylgeranyl moieties as lipid modifications were rapidly followed by the isolation and cloning of the enzymes involved in their attachment. In contrast, for the attachment of GPIs only a few obligate gene products have been identified, and many features of their biosynthesis remain to be identified [3][4]. This lag is explained by the relatively complex structure of the GPIs (*Fig. 1*) which requires a correspondingly extensive biosynthetic machinery. Much has been learned about the biosynthesis of the free GPIs through the use of radiolabeled sugars or sugar nucleotides for the metabolic labeling of intact cells or ER-derived microsomes, respectively, thus allowing the examination of very minor lipid species in their membranes. Thus, normal murine thymoma cell lines contain a pool of free GPIs (at least 3×10^6/cell) which are, as judged from their structure, ready to be attached to proteins [5]. Microsomes, if incubated with UDP-GlcNAc and GDP-mannose, make the same complete GPIs [6]. On the other hand, various mammalian mutant cell lines deficient in the biosynthesis of GPI-anchored proteins due to eight different genetic defects accumulate smaller, incomplete GPIs of various kinds, which are not seen in wild-type cells [7–10]. Similar intermediates can be made *in vitro*, when microsomes are incubated with UDP-[^{3}H]GlcNAc but limiting amounts of GDP-mannose. The structures obtained from incomplete GPI lipids can be put into order according to size by which exercise the biosynthetic *Scheme* was obtained. (This *Scheme*, although relatively likely, has still to be considered as tentative, since aberrant lipids accumulating in mutant cells cannot necessarily be considered as biosynthetic GPI intermediates, and similar though less strong reservations have to be made about lipids made in microsomes.)

Several issues in this biosynthetic scheme remain controversial:

1) All intermediates seem to be made in two forms: acylated on inositol or not, and it is presently unclear how these two forms interrelate [11][12]. It is clear, however, that, a few exceptions apart, the inositols of GPI-proteins are not acylated. The *in vitro* transfer of radiolabeled GPIs onto pre-existing microsomal GPI precursor proteins has been achieved in the trypanosomal system and has been used to demonstrate that GPIs with or without an acyl group attached to the inositol are transferred with similar efficiency [13]. Yet, in this system it cannot be excluded that the acyl gets removed from acylated GPIs prior to transfer onto proteins. At the moment, there is no way to tell whether acylinositol-GPIs or nonacylated GPIs are transferred in intact

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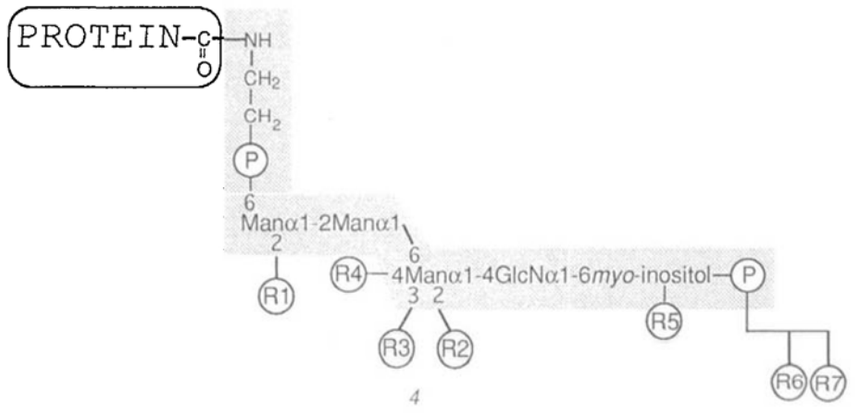
cells, and it is completely unknown at present what the role of this acyl group might be. The use of yeast secretion mutants has allowed to conclude that inositol deacylation, if it occurs on proteins, has to occur in the ER, since GPI anchors isolated from GPI-proteins trapped in the ER do not contain any acylinositol [14].

2) The sidedness of the biosynthetic reactions shown in the *Scheme* is not clear, although a few reports recently addressed this issue. *A priori*, it should be stated that the biosynthesis of the non-acylated intermediates could occur on both sides of the membrane, because all the necessary substrates are thought to occur on both sides of the membrane. UDP-GlcNAc, while produced in the cytosol, can be shuttled through an antiport system into the ER [15]. Dolicholphosphomannose also is made at the cytosolic face of the ER membrane, but it somehow can flip to the other side of the membrane and is used as the mannose donor for the mannosylation reactions required for *N*-glycosylation [16] and thus could also be used for lumenal GPI biosynthesis. On the other hand, inositol-acylation of GPIs is dependent on coenzyme A or acyl-coenzyme A which is thought to be absent from the lumen of the ER [17]. Therefore, the inositol-acylated GPIs are supposed to receive their acyl chains at the cytosolic side of the membrane. Using a mammalian thymoma line *Vidugiriene* and *Menon* [18] showed that PI-[^{3}H]GlcNAc generated by the addition of UDP-[^{3}H]GlcNAc to either microsomes or perforated cells was disposed to 70–85% at the cytosolic side of the ER membrane. The orientation of this intermediate was probed using phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme which is to big to cross the membrane and which, therefore, only can cleave lipids which are at the exterior side of cells or microsomes. Further studies by the same authors [19] in trypanosomal microsomes also indicated that bigger intermediates of GPI synthesis such as mannose$_2$-GlcNAc-PI and mannose$_3$-GlcNAc-PI are mostly cytosolic, and can rapidly get chased away in pulse-chase experiments, whereas the PI-PLC-resistant (lumenally oriented) intermediates remained constant during chase. These studies all pointed to the somewhat unexpected localization of non-acylated GPI intermediates at the cytosolic side of the ER membrane and, therefore, implied that these intermediates are made at or flipped towards the cytosolic side of the membrane. Alternatively, the GPIs could be made through separate reactions on both sides of the membrane, and this hypothesis was recently proposed to explain the fact that a cytoplasmically expressed GPI-specific phospholipase, upon transfection into *Leishmania major*, interfered with GPI anchoring of proteins but not of phosphoglycans in the transfected organism [20].

It has to be kept in mind that PI-PLCs degrade a significant amount of membrane phospholipids, therefore, might disturb the lipid bilayer and inadvertently result in lipid redistribution. The present study was undertaken, to get information on the disposition of GPIs in the yeast system and to evaluate an alternative method which avoids the pitfalls of phospholipase treatments.



GPI anchor from:	R1	R2	R3	R4	R5	R6	R7
T. brucei Variable Surface Glycoprotein (MITat.1.4)	H	H	(Galα)$_{2-4}$	H	H	$C_{14:0}$ acyl	$C_{14:0}$ acyl
Rat brain Thy-1	Manα1	$EtNPO_4$	H	GalNAcβ1	H	$C_{18:0}$ acyl	alkyl?
Leismania major PSP (surface protease)	H	H	H	H	H	$C_{16:0}$ acyl	$C_{24:0}$ alkyl
Human erythrocyte Acetylcholinesterase	H	$EtNPO_4$	H	H	$C_{16:0}$ acyl	$C_{22:4}$ acyl	$C_{18:0}$ alkyl
Human erythrocyte Decay accelerating fact.	?	$EtNPO_4$	H	H	acyl	$C_{22:4}$ acyl	alkyl?
Trypanosoma cruzi 1G7	Manα1	H	H	H	?	?	$C_{16:0}$ alkyl
Yeast proteins	(Manα)$_{1-2}$	H	H	H	H	$C_{26:0}$ acyl	$C_{26:0}$ acyl or ceramide

Fig. 1. *GPI-Anchors contain the same conserved core structure in all organisms.* The conserved core structure linking the protein to the lipid moiety is shaded. GPI-anchors differ by the type of side chains (R) and the lipid moieties. P = phosphodiester. Man = mannose, GlcN = glucosamine.

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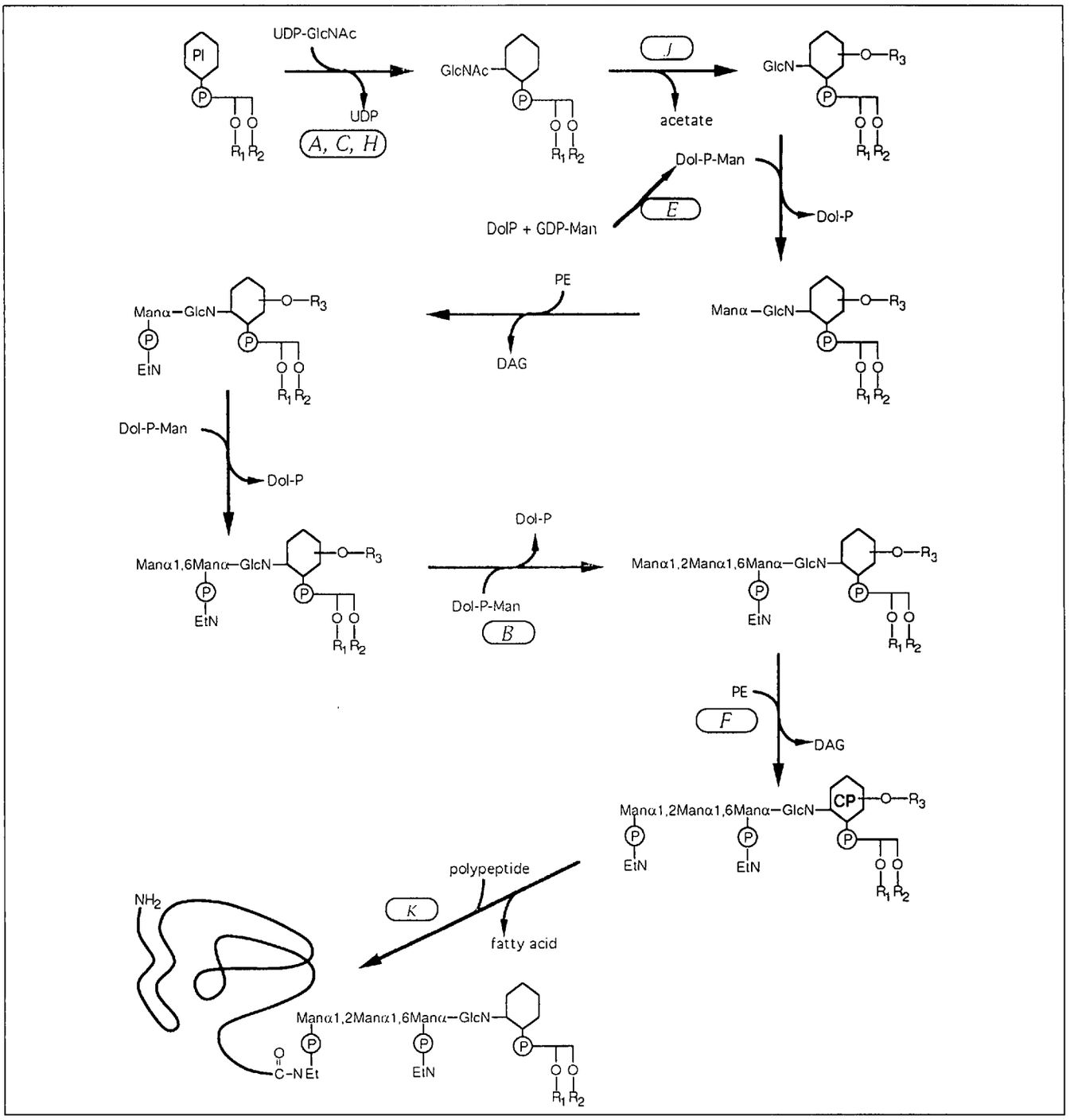
Scheme. *Hypothetical Biosynthesis Pathway for GPIs.* Biosynthesis starts out from phosphatidylinositol (PI) and leads through stepwise additions to the complete precursor (CP) ready to be attached to proteins. DolP = dolicholphosphate; Dol-P-Man = dolicholphosphomannose; PE = phosphatidylethanolamine; DAG = diacylglycerol. Boxed capital letters (*A–J*) indicate genes which are presumed to be required for the adjacent enzymatic steps, because mutations in these genes lead to the accumulation of the preceding intermediate(s) [9][10].



Results and Discussion

2,4,6-Trinitrobenzenesulfonic acid (TNBS) has been used previously as a derivatizing reagent to study membrane topology, since many membranes are impermeable to TNBS at 0° [22]. In GPIs, there are two free amino groups which potentially could react with TNBS, namely the ones of glucosamine and of ethanolamine. To evaluate the usefulness of TNBS for the study of GPIs, we first tried to react it at various pHs with a lipid extract from [^{3}H]-*myo*-inositol labeled *dpm1* cells which at 37° accumulate the intermediate GlcN-acylinositol-phosphodiacylglycerol. When monitoring the result by analytical thin layer chromatography (TLC) followed by fluorography (a radiodetection method), it appeared that the relative intensity of this early intermediate was unchanged whatever the conditions for incubation with TNBS had been. We, therefore, had to conclude that this GPI intermediate could not be derivatized on its glucosamine under any conditions. CP1 and CP2 are complete GPIs similar to the one transferred onto protein in the *Scheme*. CPs accumulate in *pmi40* mutant cells when they are labeled with

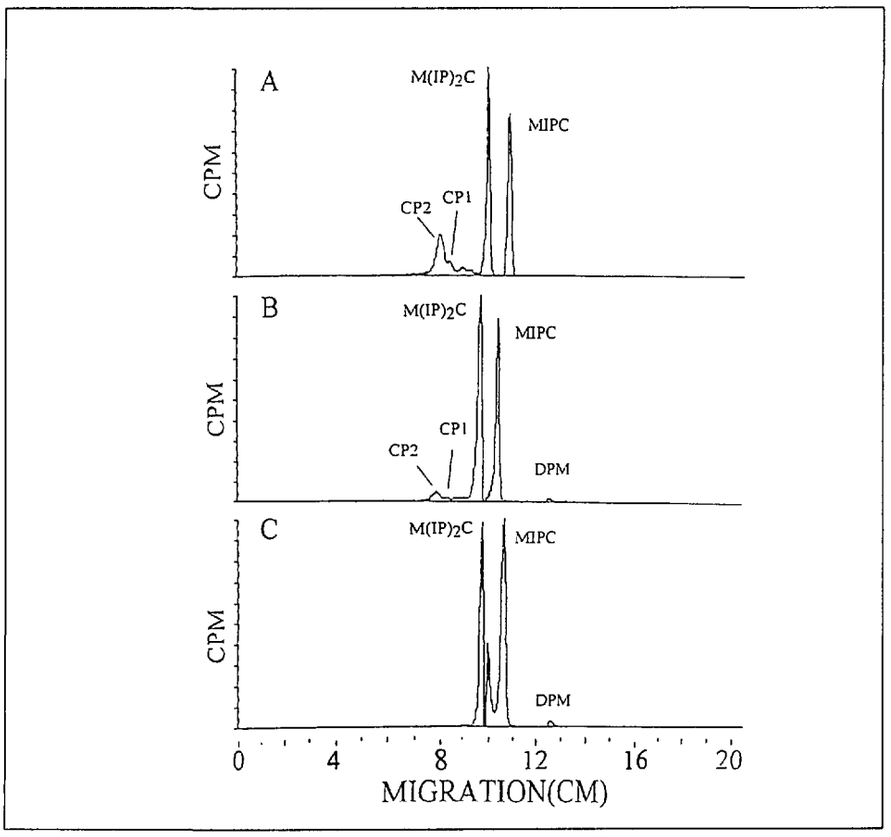


Fig. 2. *Derivatization of CP1 and CP2 in microsomes by trinitrobenzene sulfonate.* 2 x 10^8 *pmi40* cells were labeled with 100 µCi of [2-^{3}H]mannose at 37° in the presence of 10 µg/ml of cycloheximide during 50 min as described [21]. Cells were then broken by vigorous vortexing in the presence of glass beads (5 min, 0°) in 200 mM sucrose, 50 mM potassium phosphate buffer, pH 9.3. After removal of the glass beads, the microsome-containing extract was split into 3 equal aliquots and treated with buffer only (panel A), TNBS (25 mM, freshly made; panel B), or TNBS plus 0.1% *Triton X-100* (panel C) during 2 h at pH 9.3 on ice in the dark. Subsequently, reactions were quenched by the addition of a 4 fold excess of glycine, and samples were left on ice for another hour. Upon addition of glycine, the sample turned yellow as a consequence of the formation of the yellow glycyl-TNBS, thus indicating that TNBS was in large excess and had remained reactive throughout the 2 h incubation. $CHCl_3$ and CH_3OH were added to obtain a final $CHCl_3/CH_3OH/H_2O$ ratio of 10:10:3. After desalting, lipids were analyzed by TLC on silica gel using $CHCl_3/CH_3OH/H_2O$ at the ratio 10:10:3 as the mobile phase. The distribution of radioactive lipids in each TLC lane was analyzed by one- and two-dimensional radioscanning (*Berthold* LB2842) and fluorography. The diagrams represent one-dimensional distributions. MIPC = Mannosylinositol-phosphoceramide; $M(IP)_2C$ = Inositolphospho-MIPC.

[2^{3}H]mannose at 37° [21]. CP1 and CP2 were readily derivatized at pH 8.3 as well as pH 9.3, *i.e.*, they were no more detectable after tagging with TNBS while all other labeled lipids remained unchanged (not shown). (Reaction of CPs with TNBS was expected to result in the disappearance of these lipids from TLC, since the reaction products are so very polar that they cannot be expected to partition into the organic solvent phase during lipid extraction.)

Having established the conditions for the reaction with isolated CP lipids we went on to treat microsomes of [2-^{3}H]mannose-labeled *pmi40* cells with TNBS as described in the legend of *Fig. 2*. It became apparent, that under these conditions 75% of CPs were eliminated by the TNBS treatment in the absence of detergent (panel B) whereas 100% became derivatized in the presence of the detergent *Triton-X-100* which opens the tightly closed microsomes (panel C). Two minor GPI intermediates migrating between CP1 and $M(IP)_2C$ were also reduced but all other lipids were unchanged. Also, CP1 and CP2 were reduced to the same degree.

These data clearly demonstrate the usefulness of TNBS as a topological reagent for the localization of GPIs, since TNBS quantitatively reacts with CP1 as well as CP2, and since the intact membrane seems to be a permeation barrier for this reagent. ER-Derived microsomes prepared by the methods used here usually are oriented with the cytoplasmic face towards the exterior side and we, therefore, can speculate that CP lipids are displayed at both surfaces of the membrane with 75% facing the cytosol and 25% being luminal. This would be in agreement with earlier studies in the mammalian and trypanosomal systems [18][19] which also indicated a majority of intermediates at the cytosolic leaflet. However, careful studies on the membrane orientation of ER proteins in our microsomes are required to support this conclusion.

Experimental

The strains of *Saccharomyces cerevisiae* (*X2180*, *dpm1* and *pmi40*), culture media and culture conditions, radiolabeling procedures with [^{3}H]-*myo*-inositol and [2-^{3}H]mannose, lipid extraction, desalting, and thin-layer-chromatography procedures were the ones described recently [21]. 2,4,6-Tribenzenesulfonic acid was purchased from *Fluka*, Buchs, Switzerland.

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