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

Siamak Djafarzadeh and Andreas Conzelmann*

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 $3 \times 10^6$/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-[3H]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 [16] and thus could also be used for luminal 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-[3H]GlcNAc generated by the addition of UDP-[3H]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-3-GlcNAcPI and mannose-5-GlcNAcPI are mostly cytosolic, and can rapidly get chased away in pulse-chase experiments, whereas the PI-PLC-resistant (luminaly 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 PI-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.

![PROTEIN](image_url)

**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.

<table>
<thead>
<tr>
<th>GPI anchor from:</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. brucei</em> Variable Surface Glycoprotein (M1at.1.4)</td>
<td>H</td>
<td>H</td>
<td>(Gala)2-4</td>
<td>H</td>
<td>H</td>
<td>C14:0 acyl</td>
<td>C16:0 acyl</td>
</tr>
<tr>
<td>Rat brain Thy-1</td>
<td>Man1</td>
<td>EtnPO4</td>
<td>H</td>
<td>GaINAcβ1</td>
<td>H</td>
<td>C18:0 acyl</td>
<td>alkyl?</td>
</tr>
<tr>
<td><em>Leishmania major</em> PSP (surface protein)</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C16:0 acyl</td>
<td>C24:0 alkyl</td>
</tr>
<tr>
<td>Human erythrocyte Acetylcholinesterase</td>
<td>H</td>
<td>EtnPO4</td>
<td>H</td>
<td>H</td>
<td>C16:0 acyl</td>
<td>C24:0 acyl</td>
<td>C18:0 alkyl</td>
</tr>
<tr>
<td>Human erythrocyte Decay accelerating fact.</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>?</td>
<td>?</td>
<td>C16:0 alkyl</td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em> 1G7</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C16:0 acyl</td>
<td>C24:0 acyl</td>
<td>C16:0 alkyl</td>
</tr>
<tr>
<td>Yeast proteins</td>
<td>(Manα1-2)</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>C16:0 acyl</td>
<td>C24:0 acyl</td>
<td>C16:0 alkyl</td>
</tr>
</tbody>
</table>
Scheme. Hypothetical Biosynthesis Pathway for GPls. 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 GPls, 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 GPls, we first tried to react it at various pHs with a lipid extract from [3H]-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. CPI and CP2 are complete GPls similar to the one transferred onto protein in the Scheme. CPs accumulate in pmi40 mutant cells when they are labeled with...
3-5% of CPs were eliminated by the conditions. MIPC gent (panel B) whereas 100% became other labeled lipids remained unchanged as pH 9.3, were readily derivatized at pH 8.3 as well were readily derivatized at pH 8.3 as well for the reaction with isolated CP lipids reaction products are so very polar that was expected to result in the disappearance of these lipids from TLC, since the organic solvent phase during lipid extraction, they cannot be expected to partition into 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, dpni and pm40), culture media and culture conditions, radiolabeling procedures with [3H]inositol and [2-3H]mannose, lipid extraction, desalting, and thin-layer-chromatography procedures were the ones described recently [21]. 2,4,6-Trihexylresorcinol acid was purchased from Fluka, Buchs, Switzerland.

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![Fig. 2. Derivatization of CP1 and CP2 in microsomes by trinitrobenzene sulfonate. 2 x 10⁶ pm40 cells were labeled with 100 μCi of [2-3H]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 μM, 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. CHCl₃ and CH₂OH were added to obtain a final CHCl₃/CH₂OH/H₂O ratio of 10:10:3. After desalting, lipids were analyzed by TLC on silica gel using CHCl₃/CH₃OH/H₂O ratio of 10:10:3 as the mobile phase. The distribution of radioactive lipids in each TLC lane was analyzed by one- and two-dimensional radioimaging (Berthold LB2842) and fluorography. The diagrams represent one-dimensional distributions. MIPC = Mannosylinositol-phosphoceramide; M(IP)₂C = Inositolphospho-MIPC.](image-url)