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Recent progress in synthetic and biological studies of GPI anchors and GPI-anchored proteins

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Summary

Covalent attachment of glycosylphosphatidylinositols (GPIs) to the protein C-terminus is one of the most common posttranslational modifications in eukaryotic cells. In addition to anchoring surface proteins to the cell membrane, GPIs also have many other important biological functions, determined by their unique structure and property. This account has reviewed the recent progress made in disclosing GPI and GPI-anchored protein biosynthesis, in the chemical and chemoenzymatic synthesis of GPIs and GPI-anchored proteins, and in understanding the conformation, organization, and distribution of GPIs in the lipid membrane.

Keywords

Glycosylphosphatidylinositol; GPI anchor; protein; glycolipid; inositol; carbohydrate

1. Introduction

Glycosylphosphatidylinositols (GPIs) are complex glycolipids. Their attachment to the protein C-terminus is one of the most common posttranslational modifications in eukaryotic cells [1]. To date, many different GPIs have been characterized [2], all of which share the conserved construct: $\text{NH}_2\text{CH}_2\text{CH}_2\text{OP}(=\text{O})(\text{OH})-6-O\text{-Man}\alpha 1\rightarrow 2\text{Man}\alpha 1\rightarrow 6\text{Man}\alpha 1\rightarrow 4\text{GlcNH}_2\alpha 1\rightarrow 6\text{-myo-inositol-1-O-phosphoglycerolipid}$ (Figure 1) [3]. Proteins always have their C-termini linked to the phosphoethanolamine group on Man-III.

One of the most apparent functions of GPIs is to anchor surface proteins to the cell membrane (Figure 1). GPIs and GPI-anchored proteins play a vital role in many biological processes. This review summarizes the recent progress made in disclosing GPI and GPI-anchored protein biosynthesis, in the chemical and chemoenzymatic synthesis of GPIs and

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GPI-anchored proteins, and in understanding the structure and functions of GPIs using synthetic GPI derivatives.

2. GPI and GPI-Anchored Protein Biosynthesis

The common construct of GPIs and GPI-anchored proteins among different species suggests a conserved biosynthetic pathway [4], which was elucidated by using cell-free biosynthetic systems [5]. As depicted in Figure 2A, GPI biosynthesis completes in/on the endoplasmic reticulum (ER). First, a GlcNAc is added to phosphatidylinositol (PI) on the cytoplasmic surface of ER membrane. The resultant GlcNAc-PI is de-*N*-acetylated and then inositol 2-*O*-palmitoylated. GlcNAc-de-*N*-acetylation is important for the subsequent biosynthetic steps [6,7]. Thereafter, the intermediate is translocated to the ER lumen side, where Man-I is added to GlcNH₂, followed by sequential addition of Man-II and III. Once Man-I is attached, the inositol residue can be deacylated and re-acylated, suggesting that inositol acylation is reversible [8,9]. For most GPIs, the last biosynthetic step before protein attachment is the addition of phosphoethanolamine to Man-III, although sometimes additional modifications may occur at this stage [8,9].

GPI attachment to proteins also takes place on the ER lumen side via a process called GPI transamidation (Figure 2B) involving GPI transamidase (GPI-T) [10,11]. Both GPI-T and its substrates, GPI and nascent protein for GPI-attachment, are anchored to the ER membrane. Nascent protein anchor is a hydrophobic peptide at the C-terminus, which also services as the GPI attachment signal [12]. This signal is absolutely necessary for GPI transamidation, but its sequence varies in different proteins [13]. During transamidation, the GPI attachment signal is substituted with a GPI, thus GPI is always linked to the protein C-terminus. Nascent proteins have another signal peptide at their N-termini, which directs proteins to ER but is not removed during GPI transamidation [12]. After GPIs are attached to proteins, the palmitoyl group at the inositol 2-*O*-position is usually removed for the purpose of product quality control [14]. This may also facilitate GPI-anchored protein transportation from ER to the Golgi apparatus. However, this palmitoyl group can remain in some GPI-anchored proteins, such as *T. brucei* procyclic acidic repetitive protein [15], human acetylcholinesterase [16] and human CD52 antigen [17]. After GPI-linked proteins are transported to Golgi, GPIs are further modified through a process called “fatty acid remodeling” [18]. Finally, GPI-anchored proteins are delivered onto the cell surface via the trans-Golgi network.

GPI-T is a complex enzyme consisting of five different subunits, a construct conserved among different species [19]. The subunits of *Saccharomyces cerevisia* GPI-T, Gaa1p, Gpi8p, Gpi17p, Gpi16p and Gab1p, are respectively homologous to that of the human GPI-T, GAA1, GPI8, PIG-S, PIG-T and PIG-U (Figure 2C), but some difference was found between certain *T. brucei* (Figure 2C) and human GPI-T subunits. Bi and coworkers [20] proposed that: (1) Gpi8p is the centerpiece of GPI-T surrounded by and interacting with all other subunits; (2) GPI-T may have two sub-complexes: one containing Gpi8p, Gpi16p and Gaa1p, and the other Gab1p and Gpi17p; (3) the three-component complex is the core and is conserved in all species. The exact role of each subunit is not defined, but it is widely accepted that GPI8 is the catalytic subunit [21], which could hydrolyze a synthetic peptide [22]. For GPI transamidation, Cys at the GPI8 active site cleaves the GPI attachment signal to form a reactive thioester at amino acid ω and transfers its carboxyl group to GPI. Structural modeling results indicated that PIG-T/Gpi16p may mediate protein presentation to GPI8/Gpi8p [23]. PIG-T/Gpi16p may regulate GAA1 and GPI8/Gpi8p expression [24]; GAA1/Gaa1p may assist GPI presentation to GPI8/Gpi8p [25]. In theory, GPI8/Gpi8p, PIG-T/Gpi16p and GAA1/Gaa1p can constitute a catalytically functional complex [20]. Gpi17p is believed to stabilize GPI-T complex and regulate substrate selectivity [23]; Gab1p, which

contains a stretch of amino acids of fatty acid elongase, is proposed to mediate GPI presentation to Gpi8p [20].

The GPI and GPI-anchored protein biosynthetic pathway is relatively well established. Despite that, there are still questions that need clarification, such as why the biosynthesis starts on the ER cytoplasmic surface and how the biosynthetic intermediate is translocated onto the ER lumen side. In our opinion, however, a particularly intriguing topic is GPI-T, as it is not only critical for gaining insights into GPI transamidation but also a potentially useful tool in chemical biology. For example, GPI-T may be used to ligate GPIs and proteins *in vitro* for the synthesis of truly natural GPI-anchored proteins. It may also be used to ligate GPIs and various other molecules engineered to carry the GPI attachment signal. The resultant GPI conjugates should be useful for GPI research or have the targeting ability of GPIs. A major problem, however, is that GPI-T is complex and difficult to study, thus there is no report about its application yet. Any progress in this area should have a big impact.

3. Chemical Synthesis of GPI Anchors

Owing to the presence of different carbohydrates, lipids, and other functional groups, natural GPIs usually exist in heterogeneous forms that are difficult to separate. Thus, access to homogeneous and structurally well-defined GPIs and related derivatives has to rely on chemical synthesis. GPI synthesis is a significant challenge involving multiple disciplines in organic chemistry. Nevertheless, great progress has been made in the area since the first GPI total synthesis [26].

Two general strategies have been developed for GPI synthesis. One starts with GPI skeleton construction and finishes with protecting group manipulation to achieve regioselective phosphate and lipid installation. This strategy can delay the chiral phosphate introduction to a late stage. The other strategy is to install the sterically hindered phosphoglycerolipid early, e.g., following GlcN₃, to get phospholipidated intermediate **1** (Figure 3A), and then elongate the glycan. The latter strategy is convergent, so it can be used to rapidly prepare various GPIs and GPI derivatives via coupling proper oligosaccharides to the key intermediate. Moreover, it can also address problems met with late-stage phosphoglycerolipidation in the synthesis of GPIs carrying 2-*O*-palmitoylated inositol [27]. Both strategies have been used to prepare GPIs. However, due to space limitation and because GPI total synthesis has been covered by several reviews [28–30], here we will focus on recent development in diversity-oriented GPI synthesis.

Synthesis of GPI Anchors Containing Unsaturated Lipids and Other Useful Functionalities

Many natural GPIs contain unsaturated lipids that are structurally and biologically important [31]. For their synthesis, the traditional tactic to protect hydroxyl groups as benzyl ethers is unsuitable, because its deprotection is incompatible with unsaturated lipids. One attempt to solve the problem was employing benzoyl and silyl protections reported by the Nikolaev group [32,33]. Alternatively, the Guo group [34] described a strategy using the *para*-methoxybenzyl (PMB) group for global hydroxyl protection (Figure 3A). Thus, after **1** was prepared, its glycan was elongated, which was followed by further modifications. Global deprotection of **3** by a three-step, one-pot protocol gave the target GPI **4** having two unsaturated lipids. Notably, complete removal of PMB groups was accomplished with 10% trifluoroacetic acid (TFA) in < 30 minutes.

The strategy was further showcased in the synthesis of a human lymphocyte CD52 GPI anchor containing a labile polyunsaturated lipid [35]. Using the PMB group to protect hydroxyl groups also allowed for the introduction of other molecular handles. For example, clickable GPIs containing an azido and an alkynyl groups were prepared by this strategy and

effectively coupled to a fluorescent tag and an affinity tag via click reaction [36]. These GPI derivatives are valuable tools for GPI research.

Branched GPI Anchor Synthesis

To address the structural diversity of natural GPI anchors, the Seeberger group [37] developed a general synthetic strategy based on protecting group orthogonality. A key feature of this strategy is that the central mannose block, **5** or **6**, had several orthogonal protections that enabled regioselective introduction of any branching observed in nature. Its applicability was demonstrated by the synthesis of a *Toxoplasma gondii* GPI **7** with a disaccharide at the Man-I 4-*O*-position (Figure 3B) [38].

In the past two decades, numerous schemes have been developed for GPI synthesis. It is currently feasible to chemically secure all kinds of GPIs and GPI derivatives for various biological studies. With that said, GPI chemical synthesis is far from trivial, and each target must be treated as a respectful challenge that needs careful planning of the synthetic scheme and protection tactics. Thus, additional strategies or tools to facilitate GPI synthesis are definitely welcome.

4. Chemical and Chemoenzymatic Synthesis of GPI-Anchored Proteins

As all GPI-anchored proteins identified so far have their C-termini linked to the same position of the GPI core (Figure 1), it is theoretically possible to develop a generally useful method for GPI-anchored protein synthesis through site-specific reactions of GPIs and proteins. In this regard, both chemical and enzymatic methods have been explored.

The Nakahara group [39] was the first to examine GPI-linked peptide synthesis by native chemical ligation (NCL) of Cys-containing GPI analogs and C-terminal thioester of peptides. Later, the Bertozzi group [40] and the Seeberger group [41] reported the use of NCL to couple GPI analogs to proteins, showcasing its potential. Alternatively, the Guo group [42,43] reported GPI-anchored peptide/glycopeptide synthesis by regiospecific coupling of extensively protected peptides/glycopeptides with free C-termini to GPI analogs carrying a free phosphoethanolamine.

Recently, the Guo group [44–46] developed a chemoenzymatic strategy for GPI-anchored peptide/protein synthesis catalyzed by sortase A (SrtA), a *Staphylococcus aureus*-originated transpeptidase [47]. To achieve GPI-protein ligation, the target protein was engineered to bear at its C-terminus a pentapeptide, LPXTG –the sorting signal, recognized by SrtA, while the GPI anchor had a Gly residue attached to the conserved phosphoethanolamine (Figure 4). SrtA can react with the sorting signal, break the peptide bond between T and G to form a reactive thioester, and then attach the carboxyl group of T to the amino group of Gly on the GPI anchor. This reaction was proved to be effective and specific, which has been employed to couple peptides, glycopeptides, and small proteins to GPIs [48].

In contrast to the great progress in GPI total synthesis, GPI-anchored peptide/protein synthesis remains a formidable challenge. Chemical synthesis of simple GPI-peptide conjugates is feasible, but the strategy is inapplicable to large proteins, as extensive protein protection to secure site-specific GPI-protein coupling is nearly impossible. NCL and SrtA-catalyzed GPI-protein ligations are promising and may be elaborated into useful synthetic methods, but both have limitations and, more significantly, yield unnatural products. For example, NCL gives GPI-linked proteins with a Cys between the GPI and protein, which may affect the structure and function of GPI-anchored proteins. Moreover, the preparation of protein thioesters and Cys-containing GPIs is not trivial. On the other hand, SrtA-mediated GPI-protein ligation gives GPI-linked proteins with a pentapeptide between the

GPI and protein; its structural and biological impacts are unclear. Evidently, there is no practical method to access natural and homogeneous GPI-anchored proteins yet, so any development in this respect should be highly desirable.

5. Application of Synthetic GPIs to Biological Studies

As mentioned above, GPIs and GPI-anchored proteins play a critical role in many biological events [49]. However, it is impossible to discuss these findings in detail in this short review. Instead, we will focus on studies about the conformation, organization, and distribution of GPIs in the lipid bilayer, as well as other related general topics. For these studies, synthetic GPIs and derivatives should be particularly helpful.

Computer simulation combined with NMR analysis of synthetic GPIs inserted into lipid micelle suggests that they had extended conformation with a faster relative motion at the terminal mannose and decreased mobility close to the micelle [50]. This motion is oscillation relative to the membrane rather than folding. Later, it was shown that the GPI tetrasaccharide backbone forms a rather stiff rod with a residual bending elasticity and mechanical compressibility [51]. Moreover, GPIs are not only membrane-anchoring devices but also prevent transient interactions between attached proteins and the underlying lipid bilayer, thereby permitting their rapid diffusion in the lipid bilayer [40]. Clearly, GPIs have unique properties that may be critical for the proper functioning of GPI-anchored proteins. For example, delipidation of GPI-anchored proteins could induce a conformational change that can affect antibody affinity [52].

Studies have shown that GPI-anchored proteins prefer to localize in the ordered regions of cell membrane, such as the lipid rafts [53]. Accordingly, the lipid compositions of GPIs and membranes could affect GPI-membrane association and interaction. Results obtained with lipid bilayers disclosed that the membrane-anchoring domain of GPIs had a significant impact on the distribution of GPI-anchored proteins in lipid rafts [54]. GPIs and GPI-anchored proteins could also form cholesterol-dependent microdomains in the cell membrane [55], which is affected by both the lipid chain and head-group. GPI-anchored proteins with normally remodeled GPIs had elevated oligomerization tendency and immunoreactivity as compared to that with unremodeled GPIs [56]. Studies on a truncated GPI analog in monolayers revealed that phase-separation occurred above a threshold concentration owing to strong head-group interactions, whereas the GPI analog could mix with disordered lipid and induce order in a highly cooperative manner below this concentration [57]. The anchored proteins may also affect microdomain formation [58]. In resting cells, virtually all GPI-anchored proteins form transient homodimers via ectodomain protein interactions. This works with the general, low-affinity cholesterol-dependent raft-lipid interaction to generate greater rafts in steady-state cells and in response to extracellular stimulation. GPI and GPI-anchored protein buildup and microdomain formation in the cell membrane are critical for their biological functions. In addition to the examples mentioned above that delipidation and incorrect lipid structure could reduce GPI-anchored protein affinity [52] and immunoreactivity [56], raft-based lipid interaction was discovered to play a role in signal transduction by GPI-anchored protein [58]. GPIs are also involved in protein sorting, trafficking and targeting [49].

The lipid tails of GPIs do not completely extend through the lipid bilayer. Therefore, GPI-linked proteins are not so tightly anchored to the membrane as transmembrane proteins and can migrate from one cell to another to enable cell communication [59]. Moreover, GPIs can be selectively cleaved by phospholipases, such as phosphatidylinositol-specific phospholipases C and D, to release the anchored proteins. This may be a regulating

mechanism for GPI-anchored proteins [60]. On the other hand, inositol phospholipid may participate in signal transduction pathways [61].

Despite the challenge to access and study GPIs due to their complex structure and amphiphilic property, biophysical and biological investigation of GPIs has been expanding. GPIs possess many unique features that are difficult to find in simple lipids or transmembrane proteins. This may explain why nature spends much energy to synthesize and utilize these complex molecules to anchor proteins to the cell membrane. With increasing availability of synthetic GPIs and GPI analogs, studies to gain insights into the structure, function, and structure-activity relationship of GPI anchors will prosper.

6. Conclusion

GPIs represent a distinctive class of protein anchors. Their many unique properties may be critical for the proper functioning of anchored proteins. However, GPI research has been impeded by the difficult access to homogeneous and structurally defined GPIs and GPI-anchored proteins. Fortunately, progress in GPI total synthesis has made it possible to chemically secure GPIs and related derivatives. We project that one of the flourishing topics in the future will be using synthetic GPIs, especially those with fluorescent, affinity and other molecular tags, to gain insights into GPI anchorage. In contrast, access to homogeneous GPI-anchored proteins remains a significant challenge. A potential solution is using enzymes to catalyze site-specific conjugation of synthetic GPIs and recombinant proteins. For this purpose, GPI-T should be ideal, but the complex structure of GPI-T makes its expression and application difficult. Alternatively, synthetic GPI-anchored protein analogs may be used, but whether these molecules can really represent natural GPI-anchored proteins is an open question. Thus, synthetic strategies leading to natural GPI-anchored proteins are highly desirable. Finally, practical methods and tools to study GPIs and GPI-anchored proteins *in vivo* or in living cells is another future direction. Reports [62] in this area have already started to emerge.

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Highlights

Great progress has been made in revealing GPI and GPI-anchored protein biosynthesis.

Progress in GPI and GPI-anchored protein synthesis has made their access practical.

Synthetic GPIs and GPI-anchored proteins have facilitated the study of GPI biology.

Figure 2.

An outline of the GPI and GPI-anchored protein biosynthetic pathway. (A) GPI biosynthesis: After the addition of a GlcNAc unit to PI and then de-*N*-acetylation, a palmitoyl group is attached to the inositol 2-*O*-position. Next, three mannose residues are added sequentially. The final step is to attach a phosphoethanolamine group to the Man-III 6-*O*-position. The exact pathway may vary slightly in different species or cell types. (B) GPI attachment to proteins to form GPI-anchored proteins: It is realized on the lumen side of the ER membrane via GPI transamidation catalyzed by GPI-T. This reaction is regulated by the GPI attachment signal at the C-terminus of the nascent protein, and the signal peptide is replaced with a GPI anchor during transamidation. The lipids of GPI-anchored proteins are processed further before their delivery onto the cell surface via the Golgi network. (C) Schematic representations of the subunits and construct of *T. brucei* and human GPI-Ts [19].

(A) A general strategy for the synthesis of GPI anchors containing unsaturated functionalities

(B) A General strategy for diversity-oriented GPI

Figure 3.

General strategies for diversity-oriented synthesis of functionalized GPIs and GPI derivatives

Figure 4.

SrtA-catalyzed synthesis of GPI-anchored proteins. SrtA recognizes the sorting signal at the protein C-terminus, cleaves the peptide bond between T and G to form a reactive thioester with the protein, and then attaches the protein to the Gly residue on the GPI anchor to eventually afford GPI-anchored protein.