



Review Article

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Identification of ω -sites of Glycosylphosphatidylinositol Anchored Proteins

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ABSTRACT

Unique and complex post-translational modifications are present in the outer leaflet of the plasma membrane. Glycosylphosphatidylinositol (GPI) anchoring is essential for the expression of several outer membrane proteins on the cell surface. A common GPI anchor structure is constituted by glycan moiety, lipid moiety, phosphate and ethanolamine. GPI-anchored proteins (GPI-APs) are observed among eukaryotic species. Abnormal GPI anchoring of proteins is thought to cause various diseases such as paroxysmal nocturnal hemoglobinuria. Recently, many inherited GPI deficiencies (IGDs) have been reported to cause epilepsy, mental retardation, coarse facial features, and multiple organ anomalies. Diseases caused by abnormal GPI anchoring will probably continue to increase, because it is still unknown how many causative genes of IGDs are present. Therefore, in order to study these diseases, the analytical methods of GPI-APs will become important in the future. To date, many methods have been developed for analysis of GPI-APs. In this review, we attempt to summarize the present knowledge about comprehensive analytical methods of GPI-APs and introduce briefly some GPI anchor-related diseases.

Introduction

Several outer membrane proteins contain a covalently linked glycosylphosphatidylinositol anchor (GPI-anchor), which tethers the protein to the extracellular face of the plasma membrane. The common GPI core structure is ethanolamine-phosphate 6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6myo-ino-1-phosphate-lipid, and is highly conserved among eukaryotic species. All mammalian GPI anchors have a phosphoethanolamine side chain linked to the 2-position of the first α 1-4 linked mannose¹ (Figure 1). Precursors of GPI-APs contain a C-terminal GPI-attachment signal peptide that is involved in the addition of GPI anchor by the transamidase complex in the endoplasmic reticulum². GPI-anchor attachment site — the ω -site, tends to contain amino acids with small side chains, such as Gly, Ala, Ser, Asn, Asp, and Cys³. However, we revealed that other amino acids (Leu, Met and Thr) are also present at ω -sites⁴. GPI-APs are a diverse class of proteins that play critical roles in many biological processes such as immunity, inflammatory response, growth arrest, cell adhesion, lipid metabolism, fertilization, transport, and neurogenesis.

The identification of phospholipid anchored alkaline phosphatase (ALP) was first reported by Slein, M. W. & Logan, G. F. in 1963 by using phospholipase C (PLC) in mammalian cells⁵. In human cells, four types of GPI-anchored ALP have been identified to date (ALPI, ALPL, ALPP, ALPPL2). In 1988, the first complete GPI-

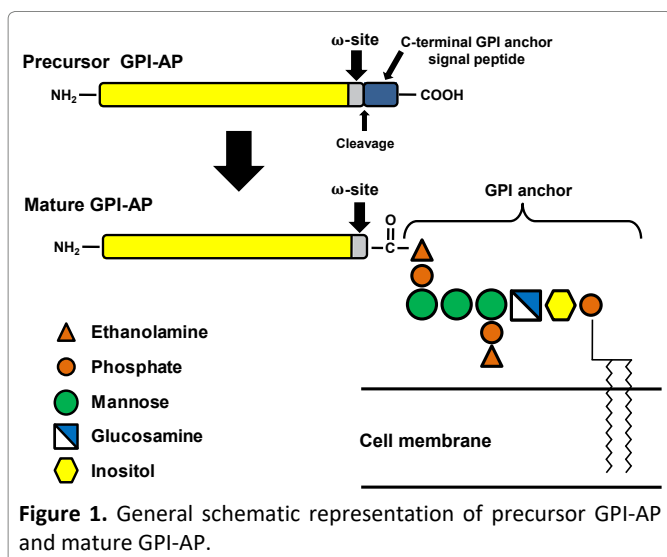


Figure 1. General schematic representation of precursor GPI-AP and mature GPI-AP.

anchor structure was obtained for *Trypanosoma brucei* variant surface glycoprotein (VSG) and rat Thy-1.

Trypanosoma brucei evades its host's adaptive immune response by VSG coat replacement⁶. To date, more than 150 proteins have been reported as a GPI-APs in mammals⁷, which play important roles in many biological processes. It is predicted that there might be 460 GPI-APs by genome analysis⁸.

GPI Anchor Related Diseases

The most famous GPI anchor related disease is paroxysmal nocturnal hemoglobinuria (PNH), which is a clonal hematopoietic stem cell disorder resulting from acquired somatic mutations in the *PIG-A* gene⁹. The *PIG* gene family encodes over 20 different proteins that are essential for the synthesis of GPI anchors. GPI-APs such as CD55 and CD59 are important complement regulatory proteins. In PNH patients, improper expression of CD55 and CD59 on blood cells leads to chronic complement-mediated intravascular hemolysis¹⁰. Eculizumab (Soliris) is a humanized monoclonal antibody that specifically binds to the complement protein C5. Eculizumab inhibits terminal complement activation and intravascular hemolysis, improving quality of life of PNH patients¹¹.

The prion protein is a GPI-AP, ubiquitously expressed throughout the body. Creutzfeldt-Jakob disease (CJD) is the most common human prion disease and a fatal neurodegenerative disorder with hallmark features of dementia, ataxia, and myoclonus¹². Prion diseases are neurodegenerative disorders characterized by changing its conformation from a cellular prion protein (PrP^C) to a scrapie isoform of the prion protein (PrP^{Sc})¹³⁻¹⁵. It was demonstrated that GPI-anchor is not required for PrP^{Sc} formation by using transgenic mice expressing a prion protein lacking the GPI-anchor¹⁶. However,

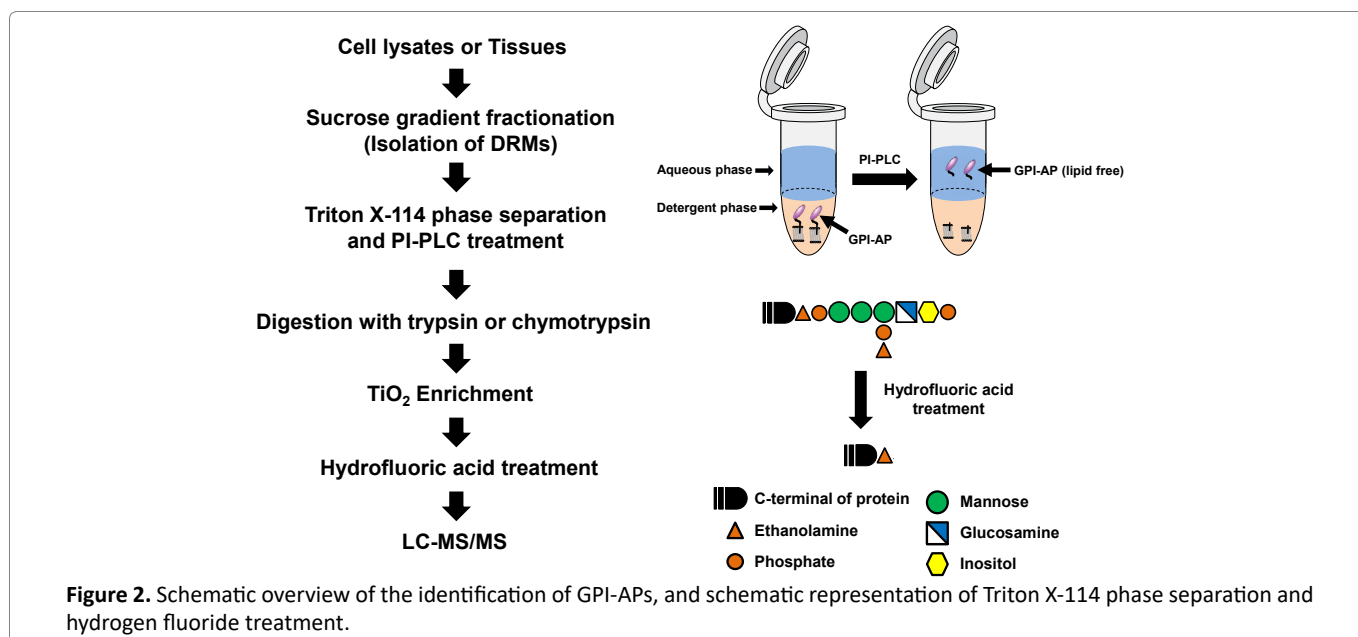
in these PrP^{Sc}-infected mice, it developed a new type of prion disease with unique clinical signs and altered neuropathology¹⁷. Campana *et al.*¹⁸ reported that GPI anchorless prion protein is secreted in a soluble form, and does not acquire scrapie-like characteristics in transfected Fischer rat thyroid cells. Nisbet *et al.*¹⁹ reported that peptides of rabbit prion proteins surrounding the ω -site prevented prion infection. Gianni *et al.*²⁰ reported that the C-terminal GPI-attachment signal peptide of PrP^C might be responsible for a genetic form of prion diseases. Sarnataro *et al.* reported that lipid rafts components might be involved in the prion disease process²¹. Although, the roles of GPI anchor in PrP^{Sc} propagation and toxicity are unclear, GPI anchor might be involved in prion disease pathogenesis.

There are at least 26 genes involved in the biosynthesis of GPI-anchors and GPI-anchoring such as *PIG* genes in the endoplasmic reticulum of mammalian cells. Recently, many inherited GPI deficiencies (IGDs) have been found using whole-exome sequencing²²⁻²⁶. Mutations of these genes (*PIGA*, *PIGL*, *PIGM*, *PIGN*, *PIGO*, *PIGP*, *PIGQ*, *PIGT*, *PIGV*, *PIGW*, *PIGY*, *PIGAP1*, *PIGAP2* and *PIGAP3*) are known to cause IGDs, and it is likely that novel IGDs will be identified in the future. Several studies summarized individual clinical symptoms of IGDs²⁷⁻²⁹. IGDs show reduced surface levels of GPI-APs or abnormal GPI-AP structure and can be diagnosed by serum alkaline phosphatase (ALP) levels and flow cytometry of granulocytes³⁰. IGDs are a group of rare diseases and the major symptoms of patients with IGDs include mental retardation and epilepsy. However, in some reported cases of IGDs, GPI-APs were expressed and localized normally³¹. This result indicates that the detail of IGDs is not yet fully understood.

Analytical Methods of GPI-APs

The photo imaging can be used to observe the lateral mobility of GPI-APs on the cell surface. It is considered that the GPI anchor provides a high lateral mobility for GPI-APs by preventing interactions between the lipid bilayer and the protein moiety of GPI-APs. A useful fluorescence reagent for a GPI-AP analysis is fluorescent-labeled inactive toxin aerolysin (FLAER) that is tagged with Alexa Fluor 488 dye and binds the glycan moiety of the GPI anchor³². FLAER staining and FLAER based flow cytometry are used to define GPI-APs on the cell surface. Combining FLAER based flow cytometry with other multiparametric flow cytometry can further improve the diagnosis of PNH and other IGDs. However, in the erythrocyte lineage, FLAER cannot be used to diagnose PNH, since erythrocytes do not possess proteolytic enzymes needed to process the Proaerolysin³³.

Because the GPI anchor is a complex structure, the identification of GPI- anchored sites by mass spectrometry



has always been considered difficult. Recently, we developed a novel method for the identification of GPI-anchored sites by combining titanium dioxide (TiO_2)-based affinity purification and hydrogen fluoride (HF) treatment. Figure 2 shows a schematic overview of strategies for identifying ω -site (GPI-anchored sites). Using this novel technique we conducted for the first time a comprehensive analysis of GPI-APs⁴. Moreover, by applying this technique, the glycan moiety of its GPI anchor can be analyzed. Using metabolic tagging, another approach, other researchers identified the GPI-APs of *Plasmodium*³⁴.

Lipid rafts are micro domains enriched in GPI-APs, cholesterol, sphingolipids, transmembrane proteins, and lipidated proteins^{35,36}. There have been several reports that lipid raft associated proteins including GPI-APs are insoluble at 4 °C in nonionic detergents, such as Triton X-100, CHAPS, and Brij 96, and able to purify these proteins by using sucrose gradient centrifugation^{37,38}. The phospholipid moiety of the GPI anchor is critical for the hydrophobicity of GPI-APs. The GPI anchor is cleaved between the phosphate moiety and the lipid moiety by treatment with a phosphatidylinositol-specific phospholipase C (PI-PLC) or phospholipase D (PLD). When subjected to temperature-induced phase separation in Triton X-114, the PI-PLC or PLD-cleaved hydrophilic forms of GPI-APs are found in the aqueous phase⁸. Elortza et al. isolated GPI-APs from *Arabidopsis thaliana* and human lipid raft associated proteins by PI-PLC and PLD treatment. This approach can indicate the presence of GPI-APs and identify ω -sites using mass spectrometric analysis. However, due to the complex structure of the GPI-anchor, comprehensive identification of the ω -sites was difficult on a proteomic scale.

The GPI anchor of VSG *Trypanosoma brucei* was isolated following aqueous HF treatment and nitrous acid deamination of the purified protein. HF treatment cleaves the phosphodiester bond of the GPI anchor moiety. Therefore, GPI anchor moiety is divided into protein moiety, glycan moiety and lipid moiety. The HF-treated GPI-APs contain only the ethanolamine of the GPI anchor (+43.0422 Da modification) (Figure 2). It is crucial to provide direct evidence for the identification of GPI-APs. In our previous study, we demonstrated that liquid chromatography-tandem mass spectrometry (LC-MS/MS) and protein database search can identify the HF-treated GPI-anchored peptides³⁹, and our study is the first comprehensive enrichment analysis of GPI-APs. Recently, TiO_2 is used to isolate phosphorylated peptides from digested peptides in phosphoproteomic analysis⁴⁰. In comparison with IMAC purification, TiO_2 is more selective for binding phosphate moiety of peptides⁴¹. In our previous study, we developed a method that uses PI-PLC treatment, HF treatment, TiO_2 -based affinity purification, and mass spectrometry. Using this method, we identified a total of 73 ω -sites derived from 49 GPI-APs. Interestingly, we found that 13 of the 49 identified GPI-APs had different amino acids at the ω -sites, yielding different protein species³. Several studies demonstrated that alteration of ω -site amino acids has a strong influence on the localization of GPI-APs. Therefore, normally, a function of GPI-APs almost certainly depends on the GPI anchor moiety in lipid rafts. The method for the identification of ω -sites could be used in a detailed analysis of GPI anchor related diseases.

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