tion, in a single patient (Fig. 1). Mosaicism was observed near the centromere of 6p, suggesting there were at least two clones with CN-6pLOH in GPI (+) cells. Both of the CN-LOH covered HLA class I and class II genes (6p21.2–6p25.3 and 6p11.2–6p25.3). HLA typing of the patient by PCR-SSOP method was *A*02:06, A*26:02, B*35:01, B*40:06, C*03:03, C*08:01, DRB1*09:01, DRB1*15:01, DQB1*03:03, DQB1*06:02, DPB1*02:01, DPB1*05:01*. The haplotype of *A*02:06-B*35:01-C*03:03-DRB1*15:01-DQB1*06:02- DPB1*05:01* was lost in 80%–90% of GPI (+) granulocytes due to 6pLOH. High frequency of CN-6pLOH in AA patients was reported by a Japanese group (7), and particular alleles including *HLA-A*02:06* were dominantly missing, suggesting 6pLOH hematopoietic stem cells escape from the immune attack mediated by cytotoxic T cells (CTLs), which may recognize unknown antigens on class I HLA molecules. HLA alleles *A*02:06, DRB1*15:01,* and *DQB1*06:02* were reported to be frequent among Japanese PNH patients (10, 11), suggesting that an immunological mechanism underlies the expansion of PNH clones. Immune mechanisms have long been hypothesized for the expansion of PNH clones with some support in the literatures. PNH clones were reported to be less sensitive to NK and T-cell killing (4, 5) or CD4 + T cells (6). GPI itself was suggested to be an autoantigen recognized by GPI-specific T cells (12, 13). GPI (+) granulocytes reflect purportedly 'normal' hematopoiesis, but our previous studies showed frequent chromosomal abnormalities and apoptotic gene expression in this 'normal' population. Based on these observations, in this patient, 80% to 90% of GPI (+) granulocytes were derived from clones that might have escaped immune attack by loss of the haplotype and therefore able to maintain nearly normal hematopoiesis for several years. The PNH clone in this patient propagated without loss of HLA alleles, supporting the idea that PNH clones are less susceptible to immune attack even when they express target antigens. HLA-restricted CTLs might have led to clonal selection of GPI (+) cells in our patient, but how the PNH cells in the same patient escaped attack is unknown. 6pLOH in the GPI (−) clone could result from mitotic recombination, but it is still to be clarified if additional CN-6pLOH endows GPI (−) cells with a comparative growth advantage under HLA-restricted immune attack. Clonal evolution in both the GPI (+) and GPI (−) cells may have caused the balanced coexistence with nearly normal blood count. Further analysis would be necessary to examine whether this phenomenon is common in other PNH patients, and assessment of both clones in the patient would be needed. In conclusion, our case supports the hypothesis that a hostile immune environment drives selection of resistant hematopoietic cell clones and indicates that clonal evolution may occur also in normal phenotype (non-PNH) cells in some cases.

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Authorship contributions

NSY was the principal investigator and takes primary responsibility for the paper; YU, SK, and NSY designed the research; YU and SK performed the laboratory work for this study; JN, YM, and YK recruited the patients in Japan and provided vital patients samples and clinical information; TK, YK, and NSY coordinated and supervised the study; YU and NSY wrote the manuscript. All authors approved the final version of the manuscript.

Conflict of interest disclosures

The authors report no potential conflict of interest.

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New insights into the functions of PIGF, a protein involved in the ethanolamine phosphate transfer steps of glycosylphosphatidylinositol biosynthesis

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PIGF is a protein involved in the ethanolamine phosphate (EtNP) transfer steps of glycosylphosphatidylinositol (GPI) biosynthesis. PIGF forms a heterodimer with either PIGG or PIGO, two enzymes that transfer an EtNP to the second or third mannoses of GPI respectively. Heterodimer formation is essential for stable and regulated expression of PIGO and PIGG, but the functional significance of PIGF remains obscure. In the present study, we show that PIGF binds to PIGO and PIGG through distinct molecular domains. Strikingly, C-terminal half of PIGF was sufficient for its binding to PIGO and PIGG and yet this truncation mutant could not complement the PIGF defective mutant cells, suggesting that heterodimer formation is not sufficient for PIGF function. Furthermore, we identified a highly conserved motif in PIGF and demonstrated that the motif is not involved in binding to PIGO or PIGG, but critical for its function. Finally, we identified a PIGF homologue from *Trypanosoma brucei* and showed that it binds specifically to the *T. brucei* PIGO homologue. These data together support the notion that PIGF plays a critical and evolutionary conserved role in the ethanolamine-phosphate transfer-step, which cannot be explained by its previously ascribed binding/stabilizing function. Potential roles of PIGF in GPI biosynthesis are discussed.

Key words: ethanolamine phosphate transferase, glycosylphosphatidylinositol, membrane protein, metabolism, PIGF, *Trypanosoma brucei*.

INTRODUCTION

Phosphatidylinositol-anchored proteins and glycans are found in evolutionarily diverse organisms including bacteria, protozoa, fungi, plants and animals. The structure of eukaryotic glycosylphosphatidylinositol (GPI) was first revealed by using a GPI-anchored variant surface glycoprotein of *Trypanosoma brucei* in 1985 [1,2]. Since then, structures of GPI-anchored proteins have been reported from many organisms and it has become clear that the core structure of protein-EtNP-6Manα1,2Manα1,6Manα1,4GlcNα1,6*myo*-inositol-phospholipid is highly conserved through the eukaryotic evolution [EtNP (ethanolamine phosphate); Man (mannose); GlcN (glucosamine)] (Figure 1). More than 20 genes involved in the GPI-anchor biosynthesis have been identified [3]. This core structure is modified by a variety of structures such as additional EtNP or mono/oligo-saccharides, but the physiological significance of various modifications in various GPI-anchored proteins remains largely unknown.

An evolutionarily conserved feature of GPI structure in mammalian and yeast cells is that an additional EtNP residue modifies both the first and second mannoses in addition to the third mannose. Phosphatidylinositol glycan anchor biosynthesis, class O (PIGO; Gpi13p in yeast) is involved in the attachment of the core EtNP on to the third mannose [4–6]. The donor of EtNP is phosphatidylethanolamine (PE) [7,8] and its EtNP moiety is transferred to GPI in a transphosphodiesterase reaction. Indeed, PIGO has conserved motifs found in phosphodiesterases and nucleotide pyrophosphatases, suggesting that it has a catalytic function. Homologues of PIGO, namely phosphatidylinositol glycan anchor biosynthesis, class N (PIGN; Mcd4p in yeast) and phosphatidylinositol glycan anchor biosynthesis, class G (PIGG; Gpi7p in yeast), mediate the transfer of EtNP to the first and second mannoses respectively [9–13]. Although the physiological significance of these additional EtNP structures are poorly understood, these steps are critical for GPI-anchor biosynthesis and trafficking. In yeast, a terpenoid lactone, YW3548, which inhibits the enzymatic activity of Mcd4p [9], blocked the addition of the third mannose, suggesting that EtNP needs to be attached to the first mannose before the third mannose transfer occurs [14]. Furthermore, mutation in the *PIGN* gene is associated with defects in forebrain development in mice and an autosomal recessive syndrome in human characterized by dysmorphic features and congenital neurological abnormalities [15–17]. *GPI7* is not an essential gene in yeast and a GPI-intermediate lacking an EtNP attachment mediated by Gpi7p can still be transferred to proteins [11,13]. Nevertheless, yeast GPI7 has been implicated in cell separation and subsequent growth of daughter cells [18]. More recently, we demonstrated in mammalian cells that the second EtNP added by PIGG is removed in the endoplasmic reticulum (ER) by a phosphodiesterase, PGAP5, soon after attachment to

Abbreviations: ALDH, acetaldehyde dehydrogenase; CHO, Chinese-hamster ovary; ER, endoplasmic reticulum; EtNP, ethanolamine phosphate; GlcN, glucosamine; GPI, glycosylphosphatidylinositol; PE, phosphatidylethanolamine; PIG, phosphatidylinositol glycan anchor biosynthesis; TM, transmembrane, WT, wild-type.

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Table 1 Primers used in the present study

Name	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
PIGF-ΔN1	AAAACTGCAGGACTACAAGGACGACGATGACAAGGTCGACGAGAACTTCTCAATATTG	ATAGTTTAGCGGCCGCTTAATTGTTCTTGTATGT
PIGF-ΔN2	AAAACTGCAGCCACCATGGACTACAAGGACGACGATGACAAGGTCGACAAACCAAATACATCCTCT	ATAGTTTAGCGGCCGCTTAATTGTTCTTGTATGT
PIGF-ΔN3	AAAACTGCAGGACTACAAGGACGACGATGACAAGGTCGACTATGGAGCACCACTGATA	ATAGTTTAGCGGCCGCTTAATTGTTCTTGTATGT
PIGF-ΔC1	AAAACTGCAGCCACCATGGACTACAAGGACGACGATGACAAGGTCGACAAAGATAACGATATCAAG	ATAGTTTAGCGGCCGCTTACGTACAGGAGATGGGCCA
PIGF-ΔC2	AAAACTGCAGCCACCATGGACTACAAGGACGACGATGACAAGGTCGACAAAGATAACGATATCAAG	ATAGTTTAGCGGCCGCTTACTGGAGACTATTCTCCCA
PIGF-ΔC3	AAAACTGCAGCCACCATGGACTACAAGGACGACGATGACAAGGTCGACAAAGATAACGATATCAAG	ATAGTTTAGCGGCCGCTTATGTTTCCAATGCCAACTC
PIGF-ΔN1ΔC1	AAAACTGCAGGACTACAAGGACGACGATGACAAGGTCGACGAGAACTTCTCAATATTG	ATAGTTTAGCGGCCGCTTACGTACAGGAGATGGGCCA
TbPIGF	ACGCGTCGACGTTCTTTGGGTAGTTATTTTCTCACTCGCT	ATAGTTTAGCGGCCGCTTATTCCACCTTTTCATCAACCTTCTCTGGT
TbPIGO	CCGGAATTCCCACCATGACCTCACGCTCTGAT	CGACGCGTTGCCAGTAACGCAGC

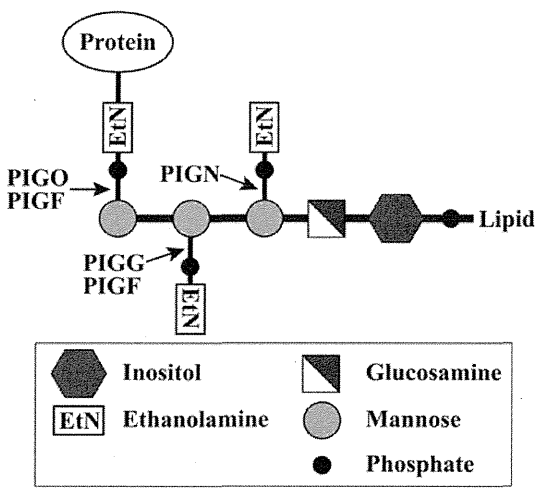

Figure 1 Structure of GPI-anchor core glycan and enzymes involved in the biosynthesis

proteins and suggested that EtNP attached to the second mannose regulates GPI-anchored protein transport in the early secretory pathway [19].

Phosphatidylinositol glycan anchor biosynthesis, class F (PIGF) is another protein involved in EtNP transfer steps of GPI biosynthesis. It is an extremely hydrophobic protein made of 219 amino acid residues in the case of human PIGF [20]. Six transmembrane (TM) domains, identified using the programs SOSUI [21] and TMHMM [22], are distributed through the entire protein. PIGF was initially identified as a protein involved in the addition of the third mannose [4]. Indeed, PIGF binds to PIGO, and PIGO quickly degrades in the absence of PIGF, indicating that PIGF is important for stable expression of PIGO [4]. Interestingly, PIGF binds not only to PIGO but also to PIGG and the stable expression of PIGG is also dependent on PIGF [10]. These observations suggested that PIGF is a critical protein that allows stable expression of the two EtNP transferases, PIGO and PIGG. Nevertheless, we know little about whether PIGF plays this stabilizing role and how the presence of PIGF is critical when co-ordination of multiple EtNP transferases is necessary. In the present paper, we provide evidence that PIGF uses distinct amino acid domains to interact with PIGO or PIGG. Furthermore, we demonstrate that PIGF plays an additional function that cannot be explained by its binding to PIGO or PIGG. Finally, whereas a protozoan parasite *T. brucei* lacks PIGG, we demonstrate the presence of a PIGF homologue, designated as TbPIGF, and its interaction with TbPIGO, indicating that evolution of PIGF is not dependent on the presence of PIGG.

EXPERIMENTAL

Cells and culturing

EL4 class F cells [20], a murine thymoma Thy-1-deficient cell-line, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Chinese-hamster ovary (CHO)-K1 cells were cultured in Ham's F12 medium supplemented with 10% FBS.

Cloning, preparation of mutant constructs and transfection

Primer sets used to create PIGF mutants are shown in Table 1. Amplified PCR fragments were digested with PstI and NotI and ligated into pMEPyori_puro-FLAG-CD59 vector to generate PIGF proteins with an N-terminal FLAG tag [23]. PIGO and PIGG with an N-terminal GST tag were generated previously [4,10]. *TbPIGF* and *TbPIGO* genes were amplified and cloned into either pMEPyori_puro-FLAG-CD59 or pMEpyori4gGST expression vector. Site-directed mutagenesis was performed as described previously [23]. For transfection of EL4 class F cells, cells were grown to a density of 3×10^6 cells/ml, and 1×10^7 cells were electroporated with 20 μg of plasmid at 1000 μF and 250 V in 0.4 mm gap cuvette. The cells were then transferred to 10 ml of Eagle's medium containing 10% FBS and cultured for 2 days. For CHO-K1 cells, 1×10^7 cells were electroporated with 20 μg of plasmid at 1000 μF and 250 V in 0.4 mm gap cuvette. The cells were then transferred to 10 ml of Ham's F12 medium containing 10% FBS and cultured for 2 days.

Fluorescence staining and FACS analysis

Surface-expressed Thy-1 was stained by incubating cells with phycoerythrin-conjugated anti-CD90.2 antibody (Becton Dickinson) for 1 h on ice. Stained cells were analysed using a FACScan cytometer (Becton Dickinson).

Immunoprecipitation and Western blotting

Washed cells were resuspended in 0.8 ml of lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% digitonin, 5 mM EDTA and protease inhibitor cocktail) and lysed for 1 h at 4 °C. The lysate was then centrifuged at 20000 ***g*** for 15 min to remove cell debris and the supernatant was incubated with glutathione–Sepharose 4B (GE Healthcare) or anti-FLAG M2 Affinity Gel

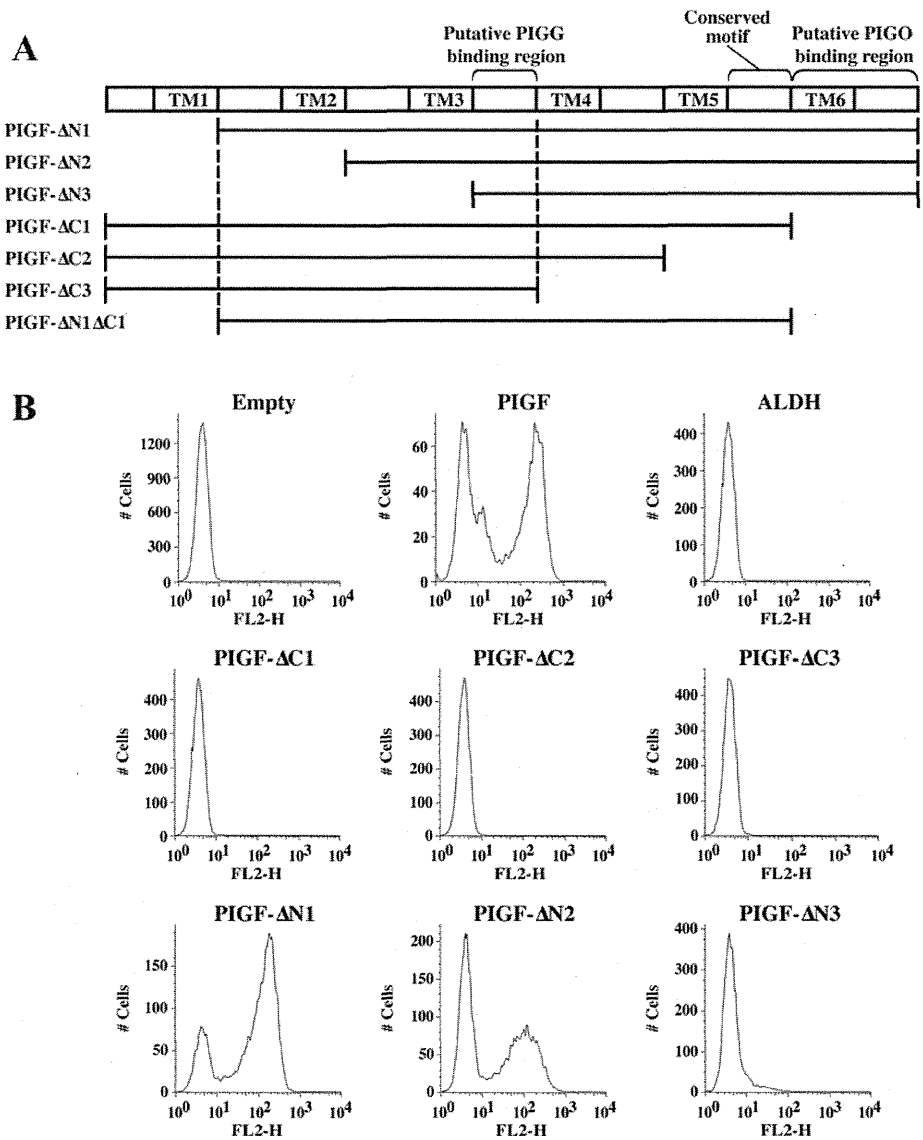

Figure 2 Complementation of PIGF-deficient cells with truncated PIGF

(**A**) Schematic representation of PIGF depicting predicted TM domains and areas of truncation in the tested constructs, binding regions and the location of the conserved motif. (**B**) EL4 class F cells were transiently transfected with the truncated PIGF constructs and tested for surface expression of Thy-1.

(Sigma). The beads were washed and bound proteins were eluted with reduced glutathione or FLAG peptide respectively. We often performed sequential pull-downs (e.g. glutathione–Sepharose pull-down and then anti-FLAG immunoprecipitation as in Figure 3) to collect unbound proteins in the initial pull-down. The eluted samples were then denatured in a sample loading buffer under reducing conditions for 1 h at 4 °C and proteins were separated by SDS/PAGE (15 % or 10–20 % gradient gel). Proteins were transferred on to PVDF membranes (Millipore) for Western blotting. Primary antibodies were mouse anti-FLAG M2 monoclonal antibody (Sigma) or goat anti-GST polyclonal antibody (GE Healthcare). For secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Cell Signaling Technology) was used and the protein bands were visualized by chemiluminescence using ECL-Plus (GE Healthcare).

RESULTS

Functional domains of human PIGF

To further examine the interaction of PIGF with its binding partners, PIGO and PIGG, a series of truncated mutants were created (Figure 2A). ORFs were created to incorporate a FLAG tag at the N-terminus for constructs PIGF-ΔN2, PIGF-ΔC1, PIGF-ΔC2 and PIGF-ΔC3 and cloned in the pMEpyori vector. In order to maintain the correct orientation for constructs PIGF-ΔN1, PIGF-ΔN3 and PIGF-ΔN1ΔC1, an ORF was generated

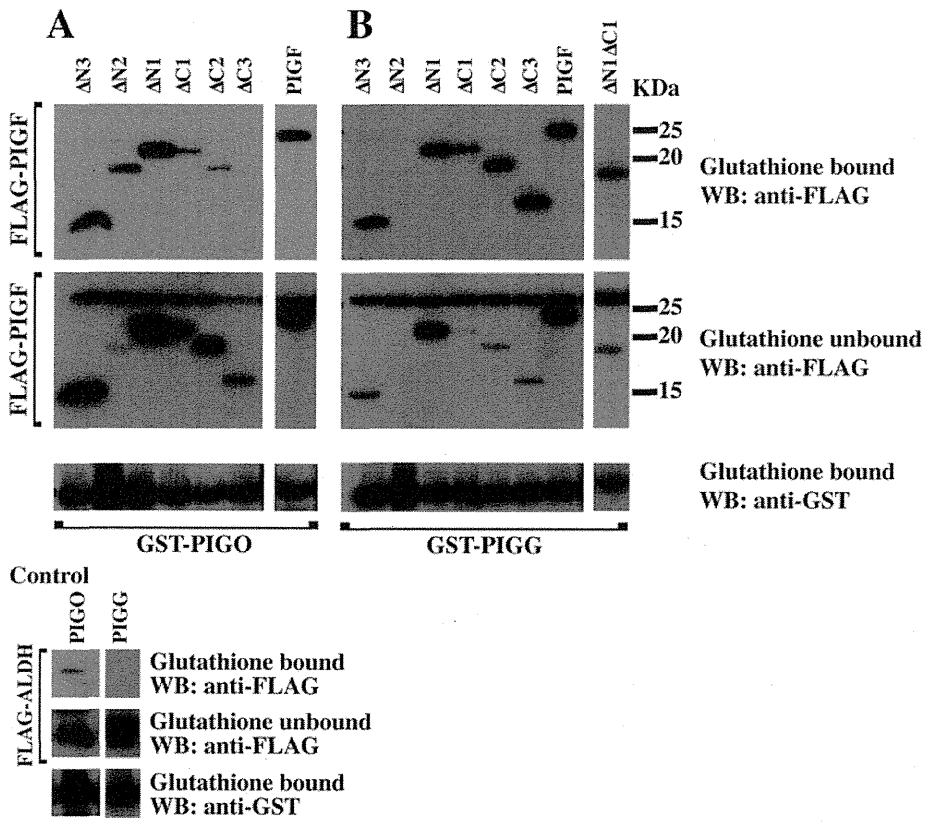

Figure 3 Binding of truncated PIGFs with PIGO and PIGG

CHO-K1 cells co-expressing WT FLAG–PIGF, truncated FLAG–PIGF or FLAG–ALDH with GST–PIGO (**A**), or GST–PIGG (**B**), were lysed in 1 % digitonin. The GST-tagged proteins in the supernatant were precipitated using glutathione beads. Unbound FLAG-tagged proteins were then precipitated with anti-FLAG–agarose. GST-tagged (bottom panels) and co-precipitated FLAG-tagged proteins (top panels) were detected by Western blot analysis using anti-GST and anti-FLAG respectively. Unbound FLAG-tagged proteins were detected using anti-FLAG antibody (middle panel). The top band constantly appearing in the middle panel is IgG light chain released during anti-FLAG immunoprecipitation.

and ligated directly into a pMEpyori containing a cleavable ER import sequence and a FLAG tag at the N-terminus [23]. To test the function of the truncated PIGF mutants, the constructs were transiently transfected into an EL4 murine thymoma cell line that lacks the surface expression of GPI-anchored proteins, such as Thy-1, due to PIGF deficiency (class F cells) [20]. Restoration of Thy-1 expression by each PIGF mutant was determined using FACS analysis (Figure 2B), and used as an indication of PIGF function. As controls, full length PIGF could restore the surface expression of Thy-1, whereas an unrelated membrane protein, acetaldehyde dehydrogenase (ALDH) had no effect on the Thy-1 expression. The removal of the C-terminal TM domain TM6 (PIGF-ΔC1) resulted in complete loss of PIGF's ability to restore Thy-1 expression in the class F cells, indicating that the C-terminal 31 amino acid residues are critical for the function or the structural integrity of PIGF. In contrast, the N-terminal two TM domains (TM1 and TM2) were not an absolute requirement for PIGF function because PIGF-ΔN1 and PIGF-ΔN2 can at least partially restore the expression of Thy-1. Further deletion (PIGF-ΔN3) abolished PIGF's ability to restore Thy-1 expression, suggesting that amino acid residues beyond position 63 carry critical residues for PIGF function. Taken together, although TM1 and TM2 are dispensable for PIGF function, the remaining C-terminal regions appear to be critical for its function.

PIGF forms heterodimers with either PIGO or PIGG and stabilizes these EtNP transferases, thereby partaking in GPI biosynthesis. We therefore wondered whether these truncated PIGF mutants lost their ability to restore Thy-1 expression because they can no longer bind to these binding partners. To test this, the truncated FLAG–PIGFs (and FLAG–ALDH as a control) were transiently co-expressed in CHO-K1 cells with GST-tagged PIGO or PIGG. The GST-tagged proteins were then immunoprecipitated with glutathione–Sepharose beads from digitonin-lysed cells and analysed for the co-immunoprecipitation of PIGF or ALDH by Western blotting. We have previously established that complexes of GPI biosynthetic enzymes are maintained in digitonin-lysed cells, but not in Nonidet P40-lysed cells [24] and successfully used ALDH, an ER-resident membrane protein as a negative control for immunoprecipitation. In the present study, we first showed that full-length PIGF binds to both PIGO and PIGG as positive controls and that ALDH binds to neither PIGO nor PIGG as negative controls (Figure 3). Figure 3(A) shows the results of co-immunoprecipitation with PIGO and indicates that the removal of the C-terminal TM domain (ΔC1, ΔC2 and ΔC3) resulted in little recovery of PIGF (upper panel). The inefficient recovery is not due to poor expression of these mutant proteins because these mutant proteins can be recovered by immunoprecipitation of unbound fractions using anti-FLAG affinity beads (lower panel). Therefore

the inability of PIGF-ΔC1, PIGF-ΔC2 and PIGF-ΔC3 to bind to PIGO is consistent with their loss of ability to restore Thy-1 expression in EL4 class F cells (see Figure 2B). These data suggest that the C-terminal TM domain is essential for PIGO binding. In contrast, the removal of the N-terminal three TM domains had no effect on PIGO binding (Figure 3A). In particular, PIGF-ΔN3 can efficiently bind to PIGO even though this construct could not restore the mutant phenotype of EL4 class F cells (see Figure 2B). These results indicate that binding of PIGF to PIGO may not be sufficient for the function of PIGF.

Because PIGF can also bind to PIGG, the inability of PIGF-ΔN3 to restore the mutant phenotype of EL4 class F cells may be attributed to its binding property to PIGG. We therefore transiently expressed the truncated FLAG–PIGFs (and FLAG–ALDH as a control) in CHO-K1 cells together with GST–PIGG. We, additionally, made a truncation mutant lacking both terminal TM domains (TM1 and TM6), designated PIGF-ΔN1ΔC1. Surprisingly, all truncated PIGFs were able to bind PIGG (Figure 3B). Because all truncated mutants contain the region between TM3 and TM4, this region may be critical for the interaction of PIGF to PIGG.

In order to clarify further the role of the C-terminal region in PIGO binding, the known PIGF amino acid sequences from various organisms were analysed. We identified a highly conserved amino acid motif consisting of the consensus PLDWxRxWQxWP (Figure 4A, red bar). To test the importance of this motif, FLAG–PIGF ORFs containing point mutations of these residues were generated and their ability to restore Thy-1 expression in class F cell was tested by FACS analysis. Only the mutations pertaining to Leu^{175} and Asp^{176} were unable to fully restore expression (Figure 4B) and point mutations W177A, Q182A, W184A and P185A were still able to restore Thy-1 expression (not shown). The importance of hydrophobicity of the leucine residue at position 175 was demonstrated by the comparison with L175K and L175A restoration effect. The effect of a positively charged lysine residue at position 175 greatly retarded Thy-1 restoration when compared with a hydrophobic alanine residue. The importance of an aspartic acid residue at position 176 was not entirely dependent on its negative charge because mutations to another negatively charged amino acid glutamic acid (D176E), positively charged amino acid lysine (D176K) or hydrophobic amino acid alanine (D176A) all showed similar reduction in surface expression of GPI-anchored proteins compared with wild-type (WT) PIGF. The combined mutation L175K/D176K had a cumulative effect and almost completely abolished PIGF's ability to restore Thy-1 expression in class F cells, indicating that these two positions are critical for the function of PIGF.

We wanted to test whether the conserved amino acids are critical for the interaction of PIGF to PIGO or PIGG. Therefore all FLAG–PIGF point mutants were co-expressed with GST–PIGO or GST–PIGG in CHO-K1 cells and immunoprecipitated using glutathione–Sepharose. All point mutants were able to bind both PIGO (Figure 4C) and PIGG (Figure 4D). Taken together, these results indicate that a conserved motif present in the C-terminal cytoplasmic loop of PIGF is functionally important, but is not involved in binding to PIGO or PIGG.

Identification of *T. brucei* PIGF and PIGO homologues

To provide insight into the evolutionary conservation of PIGF, we took advantage of the newly identified functional motif of PIGF. We used $LDW(X)_4QXWP$ as query to search the non-redundant protein sequence database at NCBI for potential PIGF homologues in other organisms. We identified a limited distribution of this motif outside of fungi, green plants and metazoans. Among these, we identified PIGF homologues in kinetoplastids (Figure 5A). The putative ORF in *T. brucei* was identified as *TbPIGF* Tb927.10.12010 (GeneID 3662199). *TbPIGF* encodes a 131-amino-acid protein with three putative TM domains. Based on amino acid sequence alignment, TbPIGF was 20.1 % identical with human PIGF.

To examine whether TbPIGF functions to bind TbPIGO, we first identified TbPIGO based on its homology with human PIGO. The putative gene *TbPIGO* (Tb927.11.5070) was cloned into a plasmid to express GST-tagged TbPIGO. This construct was transiently transfected together with a plasmid to express FLAG-tagged TbPIGF in CHO-K1 cells (Figure 5B). We then performed immunoprecipitation to examine whether TbPIGF binds to TbPIGO. We found that TbPIGF can specifically bind to TbPIGO and not to human PIGO. In contrast, human PIGF was promiscuous in that it binds to both human PIGO and TbPIGO. As a negative control, unrelated ALDH did not bind to human PIGO or TbPIGO. These data showed that TbPIGF was able to bind TbPIGO *in vivo*, further supporting a similar role for TbPIGF played in the GPI biosynthesis in *T. brucei*.

As expression and binding of TbPIGO and TbPIGF were efficient in mammalian cells, the ability of TbPIGF and TbPIGO to function in mammalian cells was tested. TbPIGF and TbPIGO were expressed alone or in combination with their mammalian binding counterpart in PIGF- and PIGO-deficient cells. The expression of these proteins failed to restore the expression of Thy-1, suggesting that they were not functional in mammalian cells (results not shown).

DISCUSSION

It was once postulated that PIGF may be an EtNP transferase [20]. However, subsequent studies demonstrated that PIGN, PIGG and PIGO are catalytic components of EtNP transferases and PIGF came to be considered as a stabilizing factor [4,10]. Our current study suggests a new additional role for PIGF, which cannot be explained by its structural role of forming heterodimers with PIGO or PIGG and stabilizing these catalytic subunits.

Two lines of evidence support our conclusion that the newly identified function of PIGF is beyond its stabilizing role. First, a truncated form of PIGF, PIGF-ΔN3, which lacks the N-terminal half of the protein, lost its functional ability to restore the surface expression of GPI-anchored proteins in a PIGF-deficient mutant (see Figure 2B), even though PIGF-ΔN3 can still bind to both PIGO and PIGG (see Figure 3). We believe that PIGF binding stabilizes PIGO and PIGG, but our data suggest that stable heterodimer formation is not sufficient for the function of PIGF. Secondly, we identified Leu^{175} and Asp^{176} as highly conserved amino acid residues critical for the function of PIGF. Double point mutation of L175K/D176K resulted in ablation of PIGF's ability to restore the surface expression of GPI-anchored proteins in a PIGF-deficient mutant (see Figure 4B). Nevertheless, the double point mutant was able to form a complex with both PIGO and PIGG, indicating again that PIGF plays a functional role that cannot be explained by binding and stabilizing PIGO and PIGG.

What is the function of PIGF? We proposed previously that the quantity of PIGF is limiting and relative activities of PIGG and PIGO are partially dictated by the availability of PIGF [10]. This scenario is based on the ability of PIGF to stably form a complex with either PIGO or PIGG, but not both, thereby controlling the relative activities of these enzymes. To consider

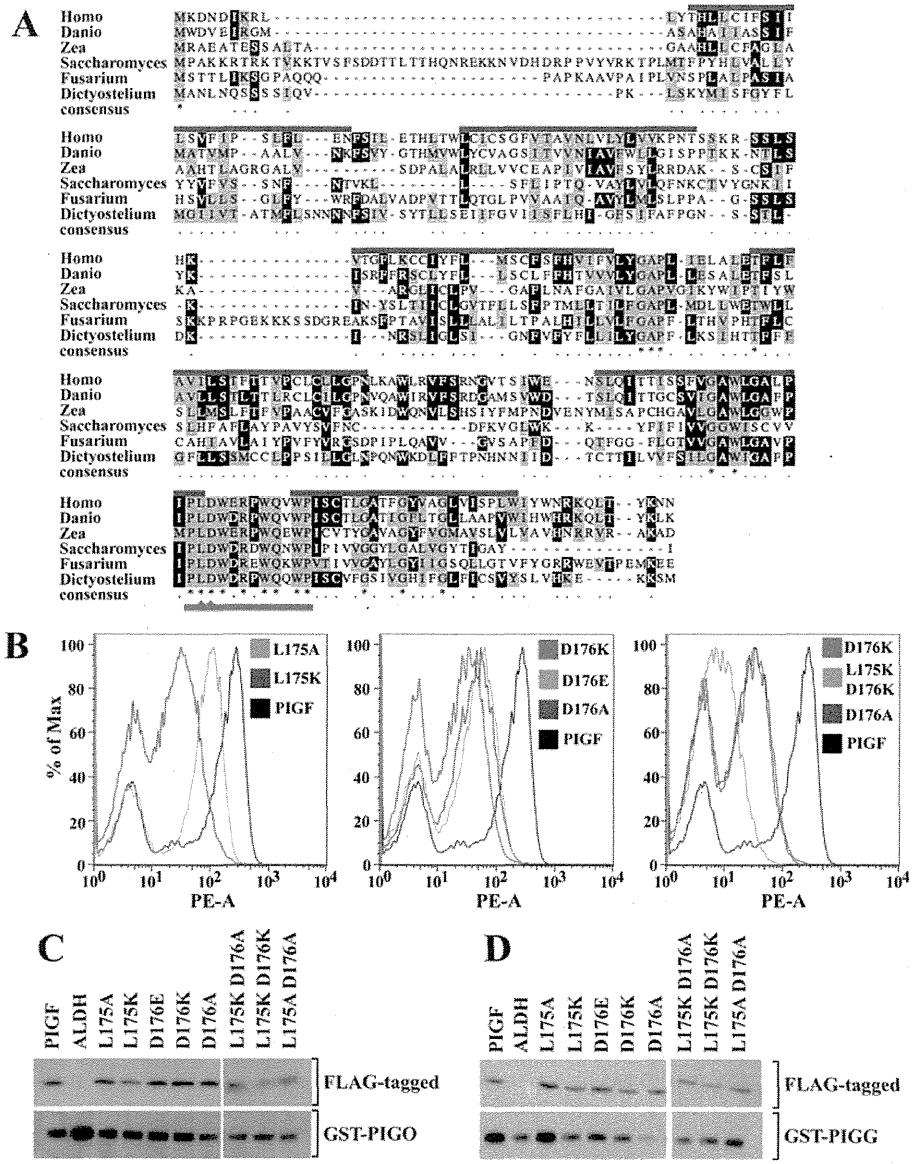

Figure 4 Evidence of functional motif in PIGF

(**A**) Alignment of amino acid sequences of PIGF homologues from *Homo sapiens* (gi|48146117), *Danio rerio* (gi|45387713), *Zea mays* (gi|525344664), *Saccharomyces cerevisiae* (gi|849212), *Fusarium pseudograminearum* (gi|408397024) and *Dictyostelium discoideum* (gi|66809485). Purple bars indicate predicted TM regions. Red bar indicates conserved motif with Leu^{175} and Asp^{176} indicated by triangles. (**B**) PIGFs containing point mutations analysed for their ability to restore Thy-1 expression in class F cells using FACS analysis. (**C** and **D**) CHO-K1 cells transiently co-expressing PIGFs containing point mutations and GST–PIGO (**C**) or GST–PIGG (**D**) were lysed in 1% digitonin. The GST-tagged proteins in the supernatant were precipitated using glutathione beads. GST-tagged (bottom panels) and co-precipitated FLAG-tagged proteins (top panels) were detected by Western blot analysis using anti-GST and anti-FLAG respectively.

this scenario further, we took advantage of another well-studied model organism of GPI biosynthesis, *T. brucei*. *T. brucei* is a protozoan parasite responsible for African sleeping sickness in human and nagana in livestock. GPI anchors in this parasite are not modified by additional EtNP residues and there is only one protein-anchoring EtNP attached to the third mannose. As such, we could only identify a PIGO homologue in the *T. brucei* genome and homologues of PIGN and PIGG are absent. If PIGF functions solely to control the relative activities of PIGG and PIGO, there is no reason for PIGF to exist in the *T. brucei* genome. It was therefore surprising that we could identify TbPIGF and demonstrate its interaction with TbPIGO (see Figure 5). Our new data suggest that evolution of PIGF is not necessarily dependent on the presence of PIGG. Taken together with the finding that PIGF functions beyond its stabilizing roles in the formation of heterodimers, we propose that PIGF plays a more fundamental role in the biosynthesis of GPI.

There are several possibilities for the additional functions of PIGF. Since it is involved in the transfer of EtNP from PE to a GPI intermediate, it is possible that PIGF is involved in the

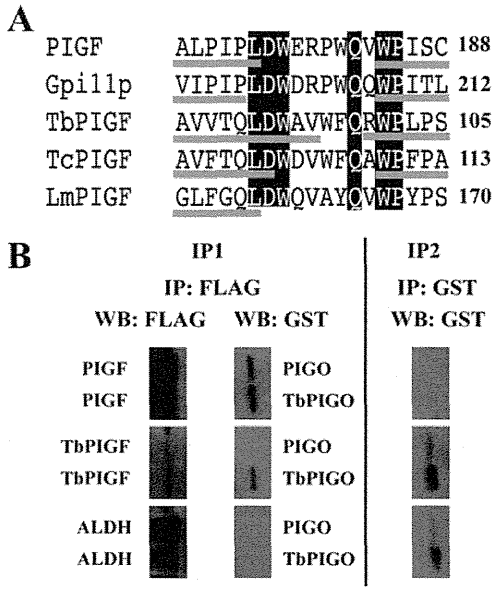

Figure 5 PIGF and PIGO homologues in *T. brucei*

(**A**) Comparison of conserved motifs from kinetoplastids with those from human (PIGF) and yeast (Gpi11p). TbPIGF (gi|71748704), TcPIGF (gi|71410645), and LmPIGF (gi|321399857: 612828-613430) indicate newly identified PIGF homologues from *T. brucei*, *Trypanosoma cruzi* and *Leishmania major*. (**B**) CHO-K1 cells transiently expressing FLAG-tagged PIGF, ALDH or TbPIGF were co-transfected with either GST-tagged TbPIGO or PIGO. Cells were lysed in 1% digitonin and the FLAG-tagged proteins in the supernatant were precipitated by anti-FLAG beads (IP1). FLAG-tagged proteins and co-precipitated GST-tagged proteins were detected by Western blot analysis using anti-GST and anti-FLAG respectively. Unbound GST-tagged proteins were immunoprecipitated by glutathione beads and detected by Western blotting using anti-GST antibody (IP2).

recruitment of these substrates to the catalytic subunit (i.e. PIGG or PIGO). We demonstrated that the aspartic acid residue in the last hydrophilic loop of PIGF is highly conserved. It is tempting to speculate that the conserved aspartic acid residue interacts with the positively charged amino group on either PE or GPI core glycan GlcN. Future studies are needed to determine its exact role in GPI biosynthesis at the molecular level.

Another focus of future research should be further analysis of heterodimer formation at the molecular level. Because both PIGF-ΔN3 and PIGF-C3 can bind to PIGG, one logical possibility is that PIGF binds to PIGG through the region between TM3 and TM4. Unfortunately, attempts to express further truncated PIGF mutants failed possibly due to the loss of protein stability. Therefore we cannot exclude an alternative possibility that two distinct binding motifs exist in PIGF for PIGG binding and one of them is sufficient for the binding of PIGF to PIGG. Whereas our current study could not pinpoint the precise amino acid residues of PIGF involved in heterodimer formation, our data demonstrate that PIGF contains two distinct binding domains: one C-terminal domain for PIGO and a second domain elsewhere for PIGG. Therefore it may be important to reconsider the possibility that PIGO, PIGG and PIGF form a heterotrimeric complex that allows efficient sequential additions of EtNP to the third and second mannose residues. Finally, we also know little about motifs in PIGO and PIGG involved in PIGF binding and more detailed studies are needed to determine the significance of interactions among these three proteins.

AUTHOR CONTRIBUTION

Matthew Stokes and Yasu Morita designed and performed experiments, analysed data and wrote the paper. Yoshiko Murakami, Yusuke Maeda and Taroh Kinoshita contributed to experimental design and data analysis.

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＝総　　説＝

知的障害とてんかんを主症状とする新しい疾患
―先天性GPI欠損症―

村上　良子　　木下タロウ

要旨　最近，先天性GPI欠損症が知能障害や乳幼児発症の難治性てんかんの原因疾患として注目を集めている．GPI（glycosyl-phosphatidyl-inositol）は150種以上のタンパク質を細胞膜につなぎとめるアンカーの役割をする糖脂質でその基本骨格は真核生物で保存されている．GPIアンカー型タンパク質の生合成とそのリモデリングに関与する遺伝子は現在までに少なくとも26個あることがわかっている．次世代シークエンサーを使ったエキソーム解析により，最近このうち12個の遺伝子を責任遺伝子とするGPI欠損症が報告されている．変異遺伝子のステップや活性の低下の程度により，症状にはバリエーションがあるが共通症状として知的障害と運動発達障害があり多くはてんかん発作を伴っている．本総説ではこの先天性GPI欠損症について，最近の知見を概説する．

見出し語　GPIアンカー，てんかん，知能障害，高アルカリホスファターゼ血症，ビタミンB6

はじめに

真核生物の細胞表面にはGPI（glycosyl-phosphatidyl-inositol）と呼ばれる糖脂質によって細胞膜に結合するタンパク質のグループ（GPIアンカー型タンパク質）が発現している．GPIの基本構造はホスファチジルイノシトール（PI），グルコサミン（GlcN），3つのマンノース（Man），2つのエタノールアミンリン酸（EtNP）から成り立っており，全ての真核生物でよく保存され，複数のステップにより合成される（図1）．ほ乳類においては現在までに150種以上のGPIアンカー型タンパク質（GPI-APs）が知られており，酵素や受容体，接着因子，補体制御因子など個体発生や神経発達，免疫機能，受精等非常に重要な働きを担っている．GPIが欠損するとこれらのすべてのタンパク質は細胞表面に発現できずに多くは細胞内で破壊されてしまうので，GPI生合成の最初のステップに必要な遺伝子*Piga*のノックアウトマウスは胎生致死になる．

Ⅰ　GPIアンカー型タンパク質（GPI-AP）の生合成とリモデリング（図2）

1. GPIアンカーの生合成

GPI-APは小胞体（ER）で蛋白部分とGPI部分が別々に合成される．GPI生合成の最初のステップは触媒サブユニットPIGAとPIGC，PIGH，PIGP，PIGQ，PIGY，DPM2の7個のタンパク質からなる酵素複合体によって担われている．その後10個のステップを経て完成したGPI-APに付加される．この反応は触媒サブユニットPIGKとPIGS，PIGT，PIGU，GPAA1の5個のタンパク質からなるトランスアミダーゼ複合体によって担われ，この酵素はGPI付加シグナルを持つタンパク質のC末端シグナルペプチドを認識して切断しGPIの末端のエタノールアミンのアミノ基と共有結合させる．このように小胞体でGPI-APに付加されるまでのステップに関わる遺伝子群を*PIG*（PhosphatidylInositol Glycan）genesと呼び，ほぼクローニングされた順にアルファベットの名前がついている．またその後の修飾に関係する遺伝子群をPost GPI Attachment to Proteins（*PGAP*）genesと呼んでいる[1]．

2. GPIアンカー型タンパク質のリモデリング

1）イノシトールの脱アシル化

GPI生合成の初期にPIGWによってGlcN-PIのイノシトールにアシル基が付加される．この反応はGPIの生合成が細胞質側から小胞体内膜側に移行した直後に起こり，その後の効率的なGPI生合成と哺乳動物においてはタンパク質と結合する末端のEtNPの付加に必要である．このアシル基はGPIアンカーがタンパク質に付加された後に小胞体でPGAP1によって除かれる．PGAP1はリパーゼモチーフ（GxSxG）を有するタンパク質で，この遺伝子の欠損細胞では小胞体からゴルジ体へのGPI-APsの輸送が大幅に遅れ，さらにゴルジ体でのリモデリングは受けず，細胞表面にはアシル基の付いた異常構造のまま発現する．そのため細菌由来のリパーゼPIPLC（phosphatidylinositol-specific-phospholipase C）による切断に抵抗性となる[2]．

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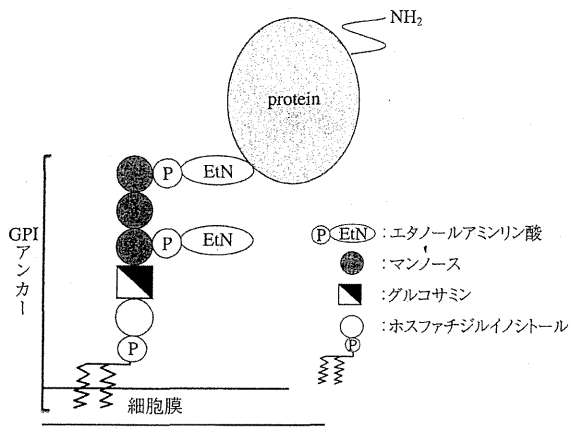

図1　GPI アンカー型タンパク質の構造

GPI の基本構造はフォスファチジルイノシトール（PI），グルコサミン（GlcN），3 つのマンノース（Man），2 つのエタノールアミンリン酸（EtNP）から成り立っており，すべての真核生物でよく保存されている．

2）GPI 糖鎖リモデリング（側鎖 EtNP の除去）

GPI 生合成過程において 3 つの Man にはそれぞれ EtNP が付加される．この反応はフォスファチジルエタノールアミンを基質とし 1 つ目の Man には PIGN が，2 つ目の Man には PIGG/PIGF 複合体が，3 つ目の Man には PIGO/PIGF 複合体が働く．このうち 2 つ目の Man に付加された EtNP は GPI アンカーがタンパク質に付加された後に小胞体で PGAP5（MPPE1）によって除かれる．PGAP5 は金属要求性のリン酸エステラーゼモチーフを有しているタンパク質で GPI-APs は PGAP5 に結合して小胞体出口部位へ運ばれさらにカーゴレセプターである p24 ファミリータンパク質と結合して小胞輸送によりゴルジ体に運ばれる．PGAP5 の欠損細胞では PGAP1 の欠損細胞と同様ゴルジ体への GPI-APs の輸送が遅れるが，ゴルジ体での脂質のリモデリングは正常に受けた後，細胞表面には EtNP が除かれない異常構造のまま発現する．

3）脂肪酸リモデリング

この反応はゴルジ体で行われ，GPI-APs が脂質ラフトに局在するために必須である．まず脂質部分の sn-2 位に付加されて

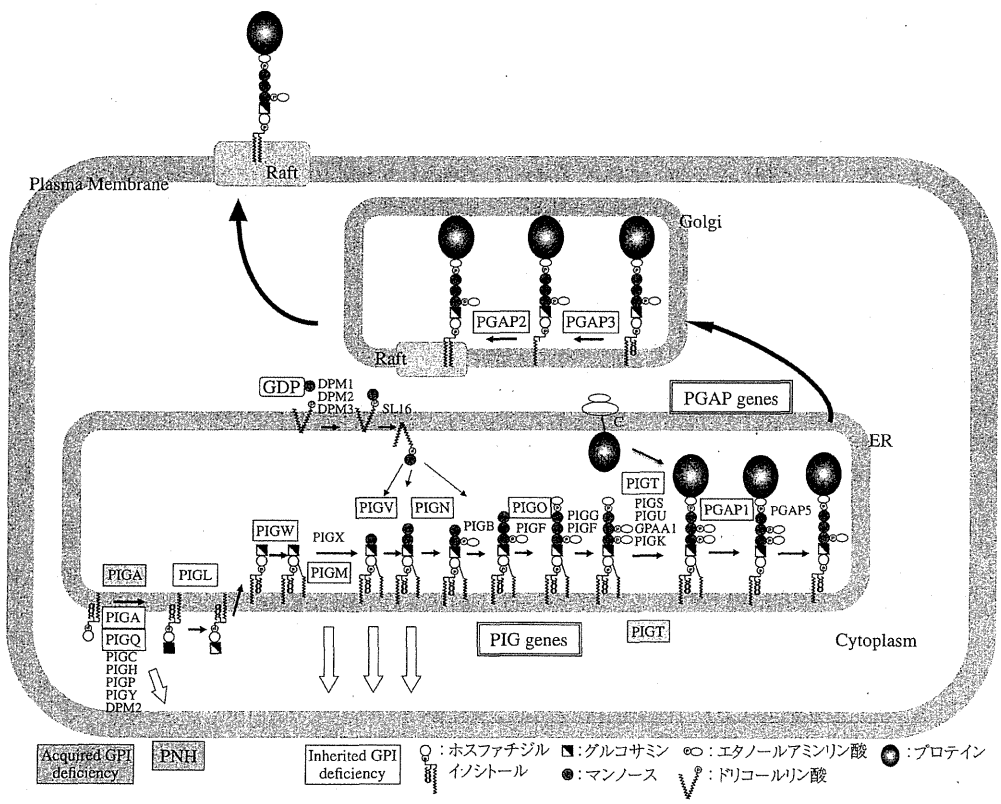

図2　GPI アンカー型タンパク質の生合成とリモデリング

GPI アンカー型タンパク質はタンパク質部分と GPI アンカー部分が小胞体（ER）において別々に合成され，GPI トランスアミダーゼ複合体が GPI 付加シグナルを持つタンパク質の C 末端シグナルペプチドを認識して切断し GPI アンカーに付加する．その後 ER およびゴルジ体で様々な修飾を受け細胞表面のラフトに局在できるようになる．白抜きの四角で囲んでいるのは現在までに報告のある先天性 GPI 欠損症，グレーの四角は後天性 GPI 欠損症（PNH）の原因遺伝子．

いる不飽和脂肪酸（アラキドン酸（C20:4）が主要）がPGAP3により除去され，飽和脂肪酸（ステアリン酸（C18:0））が付加される．この反応にはPGAP2が必須であるが，酵素ではなく制御因子であると考えられており，まだ哺乳動物で働く酵素タンパク質は同定されていない．PGAP3の欠損細胞ではGPI-APsはリモデリングを受けない異常構造のまま細胞表面に発現するが，脂質ラフトに局在できない．PGAP2の欠損細胞ではGPI-APsはsn-2位に脂肪酸が付加されないリゾ体のまま細胞表面に運ばれるが未知のリパーゼによって切断遊離され，その結果細胞表面のGPI-APsは著減する．

Ⅱ　後天性GPI欠損症である発作性夜間ヘモグロビン尿症との相違点と共通点

発作性夜間ヘモグロビン尿症（paroxysmal nocturnal hemoglobinuria;PNH）は，溶血性貧血，骨髄不全，深部静脈血栓を3主徴とする血液疾患である．溶血発作のエピソードと末梢血のフローサイトメトリーで顆粒球および赤血球表面のGPI-APsであるCD59やDAFの発現が低下あるいは欠損している細胞集団を確認することで診断される．後天的に1個あるいは数個の造血幹細胞の*PIGA*遺伝子に突然変異が起こってGPI欠損細胞となり，クローナルに増殖することによって発症する疾患なので正常細胞とGPI欠損細胞が混在していることが特徴である．血球分化にはGPI-APsは必須ではないので，クローナルに増殖している細胞の多くは完全欠損の細胞である．この異常クローンの増殖機序については未だ完全解明には至っていない．数あるGPI生合成遺伝子のうちほとんどの患者において責任遺伝子は*PIGA*である．その理由は*PIGA*のみがX染色体上の遺伝子であり，女性も発生初期のX染色体不活化後には1回の体細胞突然変異でGPI欠損細胞になるためと考えられ，実際にPNHの男女比は等しい．最近，次世代シークエンサーを用いた解析によって*PIGT*を責任遺伝子とするPNHが見つかったが，遺伝的に1本のアレルに変異があるところに，造血幹細胞において体細胞突然変異が起こり*PIGT*周辺領域の欠損が起こって発症したものであった[3]．このように今後まれではあるが他の遺伝子を責任遺伝子とするPNHが見つかる可能性がある．

一方先天性GPI欠損症（inherited GPI deficiency;IGD）においては，完全欠損は致死になるのですべて部分欠損症である．劣性遺伝形式をとり，患者は変異遺伝子のホモ接合体あるいは，複合ヘテロ接合体である．理論的にはPNHと同様1本のX染色体の変異で発症するPIGA欠損症の割合が高いと考えられる．報告例ではヘテロの女性は無症候性であり，男性のみ罹患する[4]．

Ⅲ　現在までに報告されている先天性GPI欠損症（IGD）

1. 最初に発見されたPIGM欠損症

イギリスの研究者との共同研究で解析した症例は，2家系3症例とも顆粒球ではすべてのGPI-APsが著明に減少していたが赤血球では軽微であったため溶血発作はみられず，てんかん発作と門脈血栓症が主症状であった．線維芽細胞のGPI-APsも著減していたことからIGDが疑われた[5]．患者のBリンパ芽球の解析により*PIGM*が責任遺伝子であることがわかった．PIGMは最初のマンノースを付加する酵素であるが，2家系ともに*PIGM*のプロモーターの転写因子Sp1の結合部位の1塩基の変異が原因でその結合が阻害され，ホモの変異を持つ患者では著しく*PIGM*のプロモーター活性が低下していた．Sp1はヒストンのアセチル化に関わる転写因子をリクルートする分子であるので，ヒストン脱アセチル酵素（HDAC）インヒビターであるNa butyrateの投与によって強制的にヒストンのアセチル化を起こすことによりPIGMの発現が回復しBリンパ芽球のGPI-APsの発現が完全に回復した．そこで患者にNa butyrateを投与したところ，難治のけいれん発作が治まった[6]．これらの患者には最近見つかっているIGD症例と異なり異常顔貌や奇形，知能障害などがみられない．おそらく胎生期は他の転写因子によりPIGMの発現が促進されSp1結合部位の異常による基礎的な発現低下が代償されていたのだと考えられる．一方，以下に述べる他のIGD症例に比較して血球や線維芽細胞におけるGPI-APsの低下は著明なので，血管上皮細胞のGPI-APsの発現低下等による局所の補体活性の亢進が血栓症を引き起こす原因になっている可能性がある．

2. Hyperphosphatasia mental retardation syndrome（Mabry syndrome）

古くから高アルカリホスファターゼ（alkaline phosphatase;ALP）血症と知能障害をきたす劣性遺伝の疾患が知られておりヨーロッパでは多くの患者が登録されていた．2010年にドイツの研究者が次世代シークエンサーによるエキソーム解析により，兄弟例に*PIGV*のcoding領域の遺伝子変異を発見し，我々との共同研究によりこの変異のためPIGVの活性が低下していることを示し責任遺伝子であることを証明した[7][8]．その後も同じグループとの共同研究により，同様の症状を示すPIGO欠損症[9]，PGAP2欠損症[10][11]，PGAP3欠損症[12]を報告した．また我々のスクリーニングにより国内初のGPI欠損症であるPIGO欠損症[13][14]，新規のPIGW欠損症[15]が見つかっている．PIGV欠損症はヨーロッパに多くすでに10家系以上が報告されているが，1つのアレルに共通の変異を持つ創始者効果のためと考えられる．これらの欠損症では共通症状として知的障害と運動発達障害，けいれん発作，顔貌異常が見られ，重症例では手指の末節骨や爪の低形成，難聴，心臓奇形，ヒルシュスプルング病，鎖肛等腸管の奇形，他の臓器の奇形，小脳萎縮，大脳萎縮・白質変性・髄鞘化の遅延などが見られる（図3）．このうちPGAP3欠損症以外の欠損症の症状については細胞表面のGPI-APsの発現の低下によって起こり，低下の程度によって表現型が異なるが責任遺伝子による差はあまりないと考えられる．一方PGAP3欠損症では，細胞表面の低下は顕著ではないと考えられGPI-APが脂質ラフトに局在できないために，シグナル伝達等の機能が阻害さ

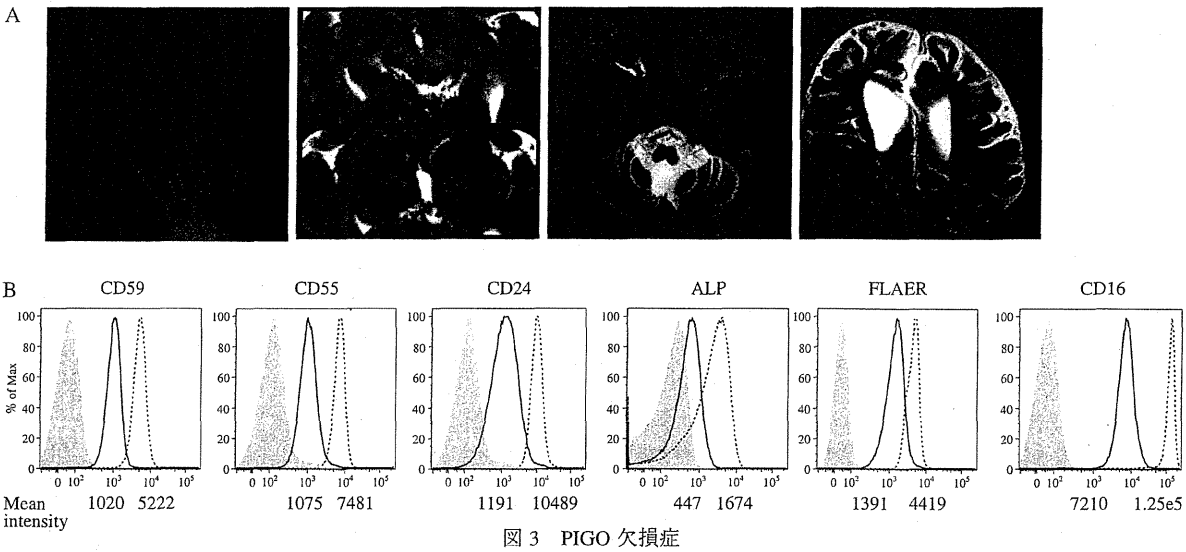

図3　PIGO欠損症

A：患者は精神発達遅滞，難治性てんかん，高アルカリフォスファターゼ血症（5959 IU/L），異常顔貌，手指末節骨の欠損（第5指），爪の低形成，ヒルシュスプルング病，ファロー四徴症をきたしていた．4歳時の手指のX線写真，4カ月時の頭部MRI（T2強調画像）では脳幹部における高シグナルが特徴的で，進行性に小脳萎縮と大脳の白質変性が見られた（共に9歳時の画像）．

B：顆粒球のFACS解析－GPIアンカー型蛋白質の発現の低下が見られた．

点線：正常コントロール，実線：患者，数値：平均蛍光強度（タンパク質発現量を示す）

（文献13）より改変）

れて起こっている可能性がある．知的障害，運動発達障害と軽いけいれん発作，時に小頭症が主症状で，PGAP3の活性がほとんどない重症例であっても発達障害は重度であるが他の臓器の奇形は伴わず手指の異常もない[12]．

3．高ALP血症の機序

責任遺伝子により機序が異なる．

1）小胞体からの分泌―PIGV，PIGO，PIGW欠損症の場合（図4）

前述したようにPIGKを触媒コンポーネントとするGPIトランスアミダーゼ複合体はGPI付加シグナルを持つ前駆タンパク質のC末端のシグナルペプチドを認識しそのωサイトで切断して酵素－基質中間体を形成し，完成したGPIアンカーに付加する．その切断のためにはトランスアミダーゼの活性化が必要でそれはGPIアンカーの複合体への結合によって起こると考えられている．その結合には少なくともマンノース1つとそれにエタノールアミンが付いていることが必要である．すなわち*PIGM*のステップより前の初期の生合成遺伝子の欠損で蓄積するGPIアンカー中間体はマンノースが付いていないので複合体に結合できず，GPI-APであるALPのシグナルペプチドの切断が起こらないためそのまま細胞内で破壊され（小胞体関連分解によると考えられる），効率よく分泌されない．一方*PIGO*等*PIGV*以降の遺伝子の欠損ではシグナルペプチドの切断が効率よく起こるが，GPIアンカーが完成されていないので付加できずそのまま分泌経路に乗って分泌され，高ALP血症になる[16]．ただしPIGN欠損症では1つ目のマンノースにEtNPが付かないが，以降の生合成は最終ステップまでゆっくり進み効率は悪いものの，シグナルペプチド切断とGPIへの付加が起こるので高ALP血症を呈さない．しかし，細胞表面のGPI-APsの発現は低下し，アンカーの構造もEtNP側鎖を欠いている．またPIGWはPIGMの前のステップでイノシトールにアシル基を付加する酵素であるが，変異によりアシル基がつかない場合，その後の反応はゆっくり進むがPIGOが担う末端のEtNPを付加する反応が起こらない．このとき蓄積するのは，PIGO欠損症で蓄積するGPI中間体のアシル基のない構造であるため，シグナルペプチド切断と分泌が起こり，高ALP血症を呈する．

逆にGPIトランスアミダーゼ複合体の必須成分であるPIGTの欠損症では切断は全く起こらず，すべてが細胞内で破壊されるため低ALP血症をきたす[17]．

2）細胞表面からの遊離―PGAP2とPGAP3欠損症の場合（図5）

前述したようにPGAP2はゴルジ体での脂質部分のリモデリング反応の際にPGAP3で除かれた不飽和脂肪酸の代わりにsn-2の位置に飽和脂肪酸を付加する働きをするタンパク質であるので，PGAP2欠損症ではGPI-APは脂肪酸を1本しか持たないリゾ体のまま細胞表面に運ばれて血清中に遊離され，細胞表面のGPI-APsの低下と高ALP血症を起こす[10)11)]．したがってPGAP2欠損症は上記のPIGV欠損症等GPI生合成遺伝子の欠損症と同様の表現型を示す．細胞表面からの遊離のメカニズムは明らかになっていないが，培養液中ではホスホリパーゼD（PLD）で切断された構造で存在するので細胞膜上あるいは遊離してからPLDによって切断されると考えら

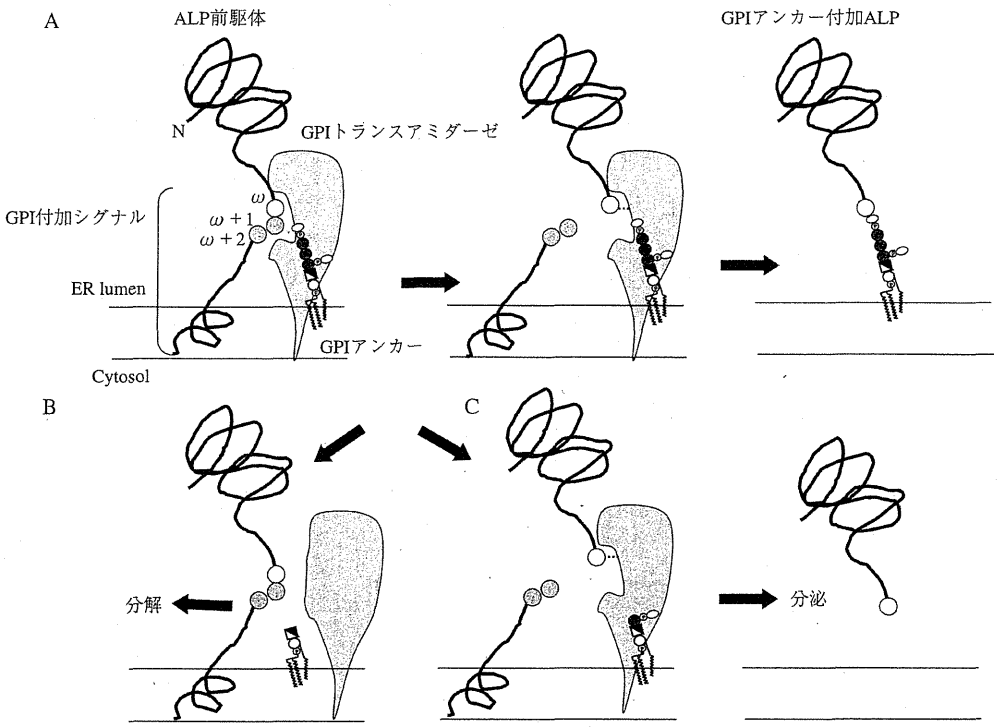

図4 高ALP血症の機序

GPIトランスアミダーゼ複合体はGPI付加シグナルを持つタンパク質のC末端のシグナルペプチドを認識しそのωサイト（切断を受けるアミノ酸）で切断して酵素－基質中間体を形成し，完成したGPIアンカーに付加する（A）．初期の生合成遺伝子やGPIトランスアミダーゼの欠損ではGPIアンカー型タンパク質であるALPのシグナルペプチドの切断が起こらないためそのまま細胞内で分解される（B）が，生合成の後期ステップの遺伝子の欠損ではシグナルペプチドの切断が効率よく起こるが，GPIアンカーが完成されていないので付加できずそのまま分泌経路に乗って分泌され，高ALP血症になる（C）．

れている．一方PGAP3欠損症ではGPI-APsはリモデリングを受けない不飽和脂肪酸を含んだ脂質部分のまま細胞表面に運ばれるので脂質ラフトに局在できない．実際PGAP3欠損症やPgap3ノックアウトマウスでは高ALP血症になることは観察されるが培養細胞では確認できず，機序は明らかになっていない．GPI局在が異常であるために生体ではリパーゼやプロテアーゼに切断されやすくなっている可能性もある．

4. 高ALP血症をきたさない先天性GPI欠損症（IGD）

1）GPI生合成遺伝子の欠損症

前述したPIGM欠損症を除くと現在までにPIGA，PIGQ，PIGL，PIGN，PIGT[4)17)~24)]欠損症が報告されている．これらはすべてGPIアンカーの生合成に関わる遺伝子なので患者の症状は細胞表面のGPI-APsの発現低下によって起こっていると考えられる．すなわちMabry syndromeと共通な症状として知的障害と運動発達障害，けいれん発作が見られ，重症例では顔貌異常，多臓器の奇形，小脳萎縮，大脳萎縮・白質変性・髄鞘化の遅延などが見られる．手指の奇形は今のところ報告されていない．PIGT欠損症では前述したように低ALP血症を来し，腸管骨の短縮や頭蓋骨の早期癒合などの骨の異常を呈する．このうちPIGN欠損症は完全欠損であっても1つ目のマンノースにEtNPが付かない異常構造のまま細胞表面に少量のGPI-APが発現する．今後症例数が増加してPIGN欠損症に特徴的な症状が明らかになるようであれば，1つ目のマンノースについたEtNPの機能的意義が解明される可能性がある．

2）PGAP1欠損症

前述のようにPGAP1欠損細胞ではGPI-APsは細胞表面にはイノシトールのアシル基が残り，脂質のリモデリングを受けない異常構造のまま発現するが，発現の低下は見られない．PGAP1ノックアウトマウスでは耳頭症（otocephaly）あるいは全前脳胞症（holoprosencephaly）といった頭部の形成異常を来たし生直後に死亡するが，まれに形成異常を示さず育つものは雄性不妊を呈する．形成異常はWntやNodalシグナルの異常，雄性不妊は細胞膜からのGPI-APsの遊離の障害が関係していると考えられている[25)26)]．一方最近PAGP1欠損症の1家系が見つかり，患者ではほとんどPGAP1の活性が認められないにもかかわらず，症状は知的障害と軽症のてんかんを呈し，顔貌は正常で奇形等も見られなかった[27)]．マウスの表現型は系統によって差が見られているので，今後症例数が増えれば特徴的な症状が明らかになると考えられる．

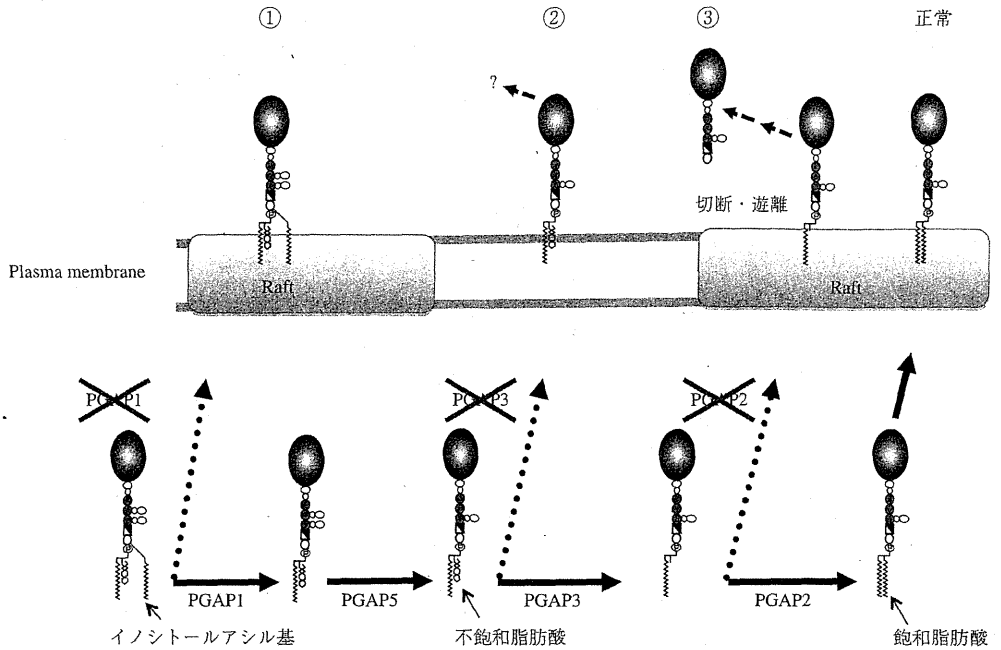

図5 GPI-APsのリモデリング

小胞体でタンパク質付加後にイノシトールのアシル基がPGAP1によって除かれる．PGAP1の欠損細胞ではGPI-APsはゴルジ体でのリモデリングは受けず，細胞表面にはアシル基の付いた異常構造のまま発現する（①）．ゴルジ体では，脂質部分のsn-2位に付加されている不飽和脂肪酸がPGAP3により除去され，PGAP2が関係する反応で飽和脂肪酸が付加される．PGAP3の欠損細胞ではGPI-APsはリモデリングを受けない異常構造のまま細胞表面に発現するが，脂質ラフトに局在できない（②）．PGAP2の欠損細胞ではGPI-APsはsn-2位に脂肪酸が付加されないリゾ体のまま細胞表面に運ばれるが未知のリバーゼによって切断遊離され，その結果細胞表面のGPI-APsは著減する（③）．

Ⅳ 症状の特徴（表1）と検査所見

GPI生合成遺伝子の欠損症ではその症状は活性の低下の程度，すなわちGPIアンカーの量に依存すると考えている．各ステップの遺伝子欠損CHO細胞に変異遺伝子を導入して正常遺伝子と比べてどの程度GPI-APsの発現を回復できるかをみる機能解析の結果は，重症度とよく相関する．最も軽症例でも知的障害は必発である．重症度が増すに従って，運動発達障害，てんかん，テント状の口などの異常顔貌，難聴，Hirschsprung病などの腸の奇形，心奇形等多臓器の奇形，時に魚鱗癬などの皮膚症状，目の異常等が見られる．乳児早期発症のてんかん性脳症である大田原症候群や早期ミオクロニー脳症，West症候群を呈する場合もある[15)20)21)]．また重症例では脳MRIで脳幹部や中脳に拡散強調やT2強調画像で高信号をきたすことが特徴である．また小脳萎縮や大脳白質変性が生後も進行する（図3A）[13)20)]．

検査所見

1）血清ALP値

高ALP血症を伴う発達障害はIGDである可能性が高い．アイソザイムは骨型，肝型共に上昇する．高ALP血症の有無は変異遺伝子のステップによることは先に述べた．このグループの患者では手指，足趾の末節骨や爪の低形成が特徴的であるが，高ALP血症が比較的軽度なPGAP3欠損症とPIGW欠損症では見られていない．一方トランスアミダーゼのコンポーネントであるPIGT欠損症ではALPが細胞内で破壊されてしまうため低ALP血症をきたし，共通の症状に加えて骨の形成異常を示す．

2）顆粒球のフローサイトメトリー（図3B）

末梢血のフローサイトメトリーで顆粒球に発現するGPI-APであるCD16の発現量の低下がIGD診断の決め手になる．重症の場合にはCD24やFLAER（GPI-APに結合するエロリジン毒素の細胞溶解能欠失変異体を蛍光ラベルしたもの）染色性等も顆粒球では低下するが，リンパ球や赤血球のGPI-APsの低下は見られない．

Ⅴ てんかん発作の発症機序と治療

てんかんの発症には神経細胞に発現するGPI-APであるALPの欠損が関係している場合があると考えられる．ALPのアイソザイムのうち組織非特異的（tissue non-specific）ALP（TNAP）の遺伝子異常は低ALP血症を来し骨の形成異常とてんかん発作を主症状とする．TNAPノックアウトマウスにおけるてんかん発作の機序が詳細に報告されている[28)]．すなわ

表1　先天性GPI欠損症の報告例と症状のまとめ

Affected Gene		PIGA (11)[a]	PIGQ (1)	PIGL (6)	PIGW (1)	PIGM (3)	PIGV (15)	PIGN (9)	PIGO (6)	PIGT (4)	PGAP1 (2)	PGAP2 (6)	PGAP3 (5)
MIM		300868	605754	280000	610275	610293	239300	614080	614749	610272	611655	614207	611801
Clinical diagnosis		Ohtahara, West syndrome, MCAHS2	Ohtahara syndrome	CHIME syndrome	West syndrome HPMRS		HPMRS1	MCAHS1	EOEE HPMRS2	MCAHS3	ID	HPMRS3	HPMRS
Age at assessment		Died (5/11)	Died at 2Y		7M	Died (1/3)	7M-17Y	Died (6/9)	0-15Y	Died at 2Y (1/4)	4Y, 2Y	3.5-28Y	4-17Y
Neurological disorder		(11/11)	(1/1)	(4/4)	(1/1)	(3/3)	(14/14)	(9/9)	(6/6)	(4/4)	(2/2)	(6/6)	(5/5)
Global developmental delay	HP:0001263[b]	Severe (9/11)	(1/1)	(4/4)	(1/1)	(1/3)	(14/14)	(9/9)	(6/6)	(4/4)	(2/2)	(6/6)	(5/5)
Motor delay	HP:0001270	Severe (9/11)	(1/1)	(4/4)	(1/1)	(1/3)	(9/9)	(9/9)	(6/6)	(4/4)	(2/2)	(5/5)	Severe (5/5)
Delayed speech	HP:0000750	No speech (11/11)		(4/4)			(9/9)	(9/9)	(6/6)	(4/4)	(2/2)	(5/6)	None (5/5)
Microcephaly	HP:0000252	(2/6) < 3rd centile		(0/6)	(0/1)		(0/9)	(0/9)	(1/6) −5.5 SD	(0/4)	(2/2)	(1/6) −4.5 SD	(3/5) −3 SD
Muscular hypotonia	HP:0001252	(8/11)			(1/1)		(9/12)	(9/9)	(6/6)	(4/4)	(2/2)	(5/5)	(5/5)
Seizures	HP:0001250	(11/11)	(1/1)	(4/4)	(1/1)	(3/3)	(11/12)	(9/9)	(4/6)	(4/4)	(1/2)	(3/6)	(4/5)
Type		Myoclonic (5/11)			Tonic spasm	Absence		Complex partial	Tonic clonic, Partial seizure, Vitamin B6 dependent	Generalized toinic clonic, Myoclonic Absence seizure	Absence	Absence Myoclonic Tonic-clonic	Generalized toinic clonic, Myoclonic
Tremor	HP:0001337	(1/3)						(7/9)			(0/2)		
Nystagmus	HP:0000639							(7/9)		(4/4)	(0/2)		
Hearing impairment	HP:0000365	(2/3)			(0/1)		(3/10)		(1/6)		(0/2)	(1/6)	(0/5)
Eye abnormality	HP:0000504	Cortical blind (3/3)		Coloboma (4/4)	(0/1)	(0/3)		Cortical blind (1/2)		Cortical blind (4/4)	(0/2)		
Abnormal facial features		(9/11)		(4/4)	(1/1)	(0/3)	(14/14)	(9/9)	(6/6)	(4/4)	(2/2)	(2/6)	(5/5)
Hypertelorism	HP:0000316	(5/7)		(4/4)	(1/1)			(1/9)	(4/6)			(1/6)	(5/5)
Long palpebral fissures	HP:0000637	(5/7)							(4/6)			(1/6)	
Broad nasal bridge	HP:0000431	(5/7)		(4/4)	(1/1)			(1/9)	(4/6)	(4/4)	(2/2)	(2/6)	(5/5)
Broad nasal tip	HP:0000455	(2/2)			(1/1)				(4/6)			(1/6)	(5/5)
Tented upper lip	HP:0010804	(5/7)			(1/1)			(3/9)	(6/6)			(2/6)	(5/5)
Micrognathia	HP:0000347	(2/6)						(4/9)					
Cleft palate	HP:0000175			(2/4)	(0/1)		(1/10)	(0/9)	(1/3)			(1/6)	(2/5)
Abnormal skeletal features					(0/1)	(0/3)				(4/4)	(0/2)		(0/5)
Craniosynostosis	HP:0001363							(2/7)	(1/6)	(2/4)			
Short arms	HP:0009824									(4/4)			
Scoliosis	HP:0002650						(1/5)			(2/4)			
Reduced mineralisation	HP:0004348									(4/4)			
Delayed bone age	HP:0003799						(1/5)			(4/4)			
Brachytelephalangy	HP:0009882	(0/11)		(0/4)	(0/1)		(13/14)	(1/9)	(4/6)			(1/6)	(0/5)
Teeth abnormality	HP:0000164	(6/11)		(4/4)	(0/1)					(2/2)			
Joint contracture	HP:0003121	(8/11)			(0/1)								
Other organ anomalies					(0/1)	(0/3)				(4/4)	(0/2)		(0/5)
Anorectal abnormalities	(anal stenosis) HP:0002025			(0/4)			(6/12)	(2/9)	(3/6)			(1/6)	
Aganglionic megacolon	HP:0002251			(0/4)			(2/14)	(0/9)	(2/6)			(1/6)	
Heart defect	HP:0001631	ASD (2/11) PDA (1/11)		VSD (1/4), TOF (1/4), TGA (1/4)			ASD (1/14)	ASD (2/7), PDA (2/7), PFO (3/7), PS (1/7)	TOF (1/6) ASD, PS (1/6)	PDA (1/4)		ASD (1/6)	
Vesicoureteral reflex or Anomalies in Urinary tract	HP:0000079	(2/4)		(1/1)			(2/5)	(4/9)		(4/4)			
Skin abnormalities					(0/1)	(0/3)					(0/2)		
Deep plantar groove	HP:0001869							(6/7)					
Skin psoriasis, Ichthyosis	HP:0008064	(3/11)		(4/4)									
Clinical Test													
Decreased expression of GPI-APs on patients' cell		(8/8) PMN		(1/1) B lymphoblast, Fibroblast	(1/1) PMN	(2/2) PMN	(2/2) PMN	(2/2) Fibroblast (2/2) PMN	(2/2) PMN	(2/2) PMN	(0/2) B lymphoblast PIPLC resistant		(1/2) PMN
Hyperphoshatasia	HP:0003155	Fluctuating mild elevation (4/9)			(1/1)	(0/3)	(14/14)	(0/2)	(4/6)	(4/4) Hypophosphatasia	(0/2)	(4/4)	(5/5)
MRI abnormality						(0/3)				(3/4)	CT, Brain atrophy		
Thin corpus callosum	HP:0002079	(6/11)						(1/4)					(1/5)
White matter immaturity	HP:0002500	(9/11)	(1/1)		(1/1)			(2/4)	(1/1)				
Restricted diffusion pattern					(1/1)				(1/1)				
Cerebellar atrophy	HP:0001272	(3/11)						(1/4)	(1/1)	(2/4)			
Others		Hepatosplenomegaly (4/4), Iron overload (4/4)				Portal vein thrombosis (2/3)		Large ears (5/7)			Large ears (2/2)		
Reference		4), 18), 19), 20)	21)	22)	15)	5), 6)	7), 8)	23), 24)	9), 13), 14)	17)	27)	10), 11)	12)

MCAHS: multiple congenital anomalies-hypotonia-seizures syndrome, CHIME: colobomas of the eye, heart defect, ichthyosiform dermatosis, mental retardation, and ear defects, HPMRS: hyperphosphatasia mental retardation syndrome, ID: intellectual disability, ASD: atrial septum defect, PDA: patent ductus arteriosus, VSD: ventricular septal defect, TOF: tetralogy of Fallot, TGA: Transposition of great arteries, PS: pulmonary stenosis, PMN: polymorphonuclear leukocyte

a) Numbers in parentheses: number of affected indivisuals, b) Human Phenotype Ontology ID

表2 先天性GPI欠損症の臨床症状

多くは知的障害，運動発達障害，てんかん発作を伴う．（時に家族性に見られる）
新生児期，乳児期早期発症の難治性てんかん（大田原症候群・West症候群）
顔貌異常（両眼解離・幅の広い鼻梁・長い眼裂・テント状の口・口唇，口蓋裂）
手指，足趾の異常（末節骨の短縮・爪の欠損，低形成）
その他の奇形（肛門，直腸の異常・Hirschsprung病・水腎症・心奇形など）
難聴・眼，視力の異常
皮膚の異常（魚鱗癬など）
筋緊張の低下，四肢の短縮，関節拘縮
高アルカリフォスファターゼ血症
低アルカリフォスファターゼ血症

ちALPは細胞表面で，ピリドキサールリン酸を脱リン酸化して細胞内に取り込める形のピリドキサールにし，細胞内に入ったピリドキサールは再びリン酸化されてピリドキサールリン酸となり，抑制性ニューロンにおいてγ-アミノ酪酸（GABA）合成酵素の補酵素として働く．細胞膜上にALPが発現しないと細胞内のピリドキサールリン酸が不足しGABA合成が抑制される結果けいれん発作がおこると考えられる．実際，細胞内のピリドキサールを補うために，患者にビタミンB6（ピリドキシン）の投与を行ったところけいれん発作が消失した[13]．一方ビタミンB6が効かない症例もあり，ALP以外のGPI-APsも多く神経細胞には発現しているので，それらの発現低下がてんかん発作に関与していると考えられる．

Ⅵ 治療の可能性

多くの症例は，大脳の白質変性や小脳萎縮が進行し，発語の消失など退行を示す．将来的には生後すぐに血液検査でスクリーニングして診断し，GPIアンカー生合成を促進する薬を投与すれば，けいれん発作等，神経症状の進行を止めることができる可能性がある．前述したように患者の遺伝子異常はほとんどが部分欠損症であり，変異を持ったcDNAでも強いプロモーターで発現させると，多くの場合正常のcDNAと同様にGPI-APsの発現を回復させることができる．今後化合物のライブラリーを用いてハイスループットにGPIアンカー生合成を促進できる化合物をスクリーニングする系を作りたいと考えている．また症状の原因となっているGPI-APが同定できれば，補充療法も可能になる．前述したビタミンB6による治療もその一つであり，葉酸受容体がGPI-APであることから，フォリン酸の投与が奏功する可能性もある．

Ⅶ 今後の展望

表2にあげた患者を対象に海外，国内の多くの症例を集積し，詳細な症状の観察と検査所見をもとに疾患概念を確立させると共に診断基準，国内外共通の患者データーベースを作成することを目標としている．

GPI生合成遺伝子の欠損症では，変異による活性低下の程度によって症状にバリエーションがある．GPIアンカーの量が制限されているときには，それに付加されるタンパク質の優先度はタンパク質のC末端のGPI付加シグナルの配列に依るとされている．すなわち少しの活性の低下で発現が減少するタンパク質と下がりにくいタンパク質がある．活性がどこまで下がるとどのような症状がでるのか，その症状はどのGPI-APsの低下に起因するのか，そのタンパク質の生理的な機能は何なのか？ iPS細胞を使った細胞レベルの解析やモデルマウスの解析により神経症状の機序の解明を目指したいと考えている．

著者の利益相反：本論文発表内容に関連して開示すべき事項なし．

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Inherited GPI deficiencies: a new disease with intellectual disability and epilepsy

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Glycosylphosphatidylinositol (GPI) is a glycolipid, which anchors 150 or more types of proteins to the cell surface. There are at least 26 genes involved in the biosynthesis and transport of GPI-anchored proteins (GPI-APs). Many inherited GPI deficiencies (IGDs) have been recently found using whole-exome sequencing. Patients with IGD have only a partial deficiency because complete GPI deficiency causes embryonic death. The major symptoms of IGDs include intellectual disability, epilepsy, coarse facial features, and multiple organ anomalies. These symptoms vary in severity depending upon the degree of the defect and/or position in the pathway of the affected gene. We clarified a mechanism of hyperphosphatasia, which is characterized by elevated release of tissue-nonspecific alkaline phosphatase. Hyperphosphatasia is observed in some patients with IGDs, such as hyperphosphatasia mental retardation syndrome or Mabry syndrome, caused by mutations in genes in the later stage of GPI biosynthesis. The possibility of IGD should be considered in patients with seizures and intellectual disability. The presence of hyperphosphatasia is strong evidence of IGD. Flow cytometric analysis of GPI-APs on granulocytes is also useful for the detection of IGD.

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Gene Section

Review

IL17A (interleukin 17A)

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Abstract

Interleukin-17A (IL17A), a characteristic cytokine produced by the T helper 17 cells (Th17 cells), can form either a homodimer or a heterodimer with IL17F.

It is produced not only by Th17 cells, but also by cytotoxic $CD8^+$ T cells (Tc17 cells), γδ T cells, invariant natural killer T cells (iNKT cells), lymphoid tissue inducer cells (LTi cells), and other hematopoietic and non-hematopoietic cells. During development, these cells exhibit flexible or plastic features distinct from those of Th1 and Th2 cells. IL17A plays important roles in the pathogenesis of autoimmune diseases and in the host defenses against bacterial and fungal infections.

Expression of IL17A and its related factors, as well as the infiltration of IL17A-producing cells into the tumor microenvironment, has been implicated in anti-tumor or pro-tumor effects in various cancers.

Keywords: Th17 cells, RORγt, STAT3, IL23, TGFβ, inflammation

Identity

Other names: CTLA8, IL-17, IL-17A, IL17

HGNC (Hugo): IL17A

Location: 6p12.2

Local order: pter - PKHD1 (polycystic kidney and hepatic diseases 1) - MIR206 (microRNA 206) - MIR133B (microRNA 133b) - **IL17A** - IL17F (interleukin 17F) - SLC25A20P1 (solute carrier family 25, member 20 pseudogene 1) - MCM3 (minichromosome maintenance complex component 3) - centromere.

DNA/RNA

Note

IL17A was initially identified in a subtractive hybridization screen of a rodent T cell library as mouse cytotoxic T lymphocyte-associated antigen 8 (mCTLA8) (Rouvier et al., 1993), but is now recognized as a characteristic cytokine of the Th17 cell subset, which has effector functions distinct from those of Th1 and Th2 cells (Korn et al., 2009; Kurebayashi et al., 2013).

Description

The IL17A gene spans a region of 4252 bp, consisting of three exons.

Transcription

The transcript is 1859 bp and has a 45 bp 5' UTR, a 468 bp coding sequence, and a 1346 bp 3' UTR.

Pseudogene

No pseudogenes homologous to this gene exist elsewhere in the genome.