mM final concentration), and the culture was grown for an additional 2 hours. The cells were pelleted and resuspended in buffer A [50 mM tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 5 mM DTT, and phenylmethylsulfonyl fluoride (PMSF; 50 μg/ml)]. After we froze and then thawed the cells, they were lysed by sonication on ice. Triton X-100 and NaCl were added to final concentrations of 1% and 0.3 M, respectively, and the lysate was incubated for 30 min at 4°C on a rotating wheel. The cellular extract was pelleted at 12,000 rpm for 15 min, and the supernatant was dialyzed extensively (4 × 500 ml) against buffer A containing 150 mM NaCl at 4°C. The dialyzed sample was loaded on a glutathione-Sepharose (Pharmacia) affinity column equilibrated with buffer A and washed several times. To cleave the RBP9-27 protein from the GST portion, we washed the column extensively with buffer A containing 2.5 mM $CaCl_2$ but no EDTA or PMSF. The Sepharose beads were then incubated with bovine thrombin (50 μg/ml) in the same buffer for 30 min at room temperature and were washed five times with 1 ml of buffer A to elute the cleaved protein. The thrombin-cleaved RBP9-27 protein was dialyzed against storage buffer [20 mM tris-HCl (pH 8.0), 0.2 mM EDTA, 150 mM NaCl, 5 mM DTT, PMSF (50 μg/ml), and 20% glycerol] and stored at −70°C. We determined RBP9-27 protein to be >95% pure.

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21. RNA-protein complexes were measured by a filter-binding assay. Thrombin-cleaved RBP9-27 (40 pM) was incubated with 0.1 pM of [^{32}P]UTP-labeled RRE330 RNA, RREΔ345 RNA, or poly(I-C) RNA (Pharmacia) in the presence of increasing amounts of unlabeled competitor [*E. coli* tRNA (Boehringer Mannheim) or as specified] in 50 μl of binding buffer 2 [10 mM tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 2 mM DTT, 0.01% bovine serum albumin (BSA), and RNasin (20 U/ml) for 20 min at room temperature. The complexes were spotted on nitrocellulose filters (25-mm diameter, 0.45-μm pore size; Schleicher Schuell, Keene, NH), washed extensively with binding buffer 2, and air-dried for 30 min. The bound radioactivity was measured in a scintillation counter.
22. P. Constantoulakis *et al.*, unpublished data.
23. Eukaryotic expression plasmids for RBP9-27 and the two related interferon-inducible proteins 1-8U and 1-8D were constructed as follows. Total RNA was isolated from HeLa cells after induction with IFN-α, reverse transcribed, and PCR amplified as described (*19*). The primers used were as follows: 5′-TTCCCCAAAGCCAGAAGATGCA-3′ (sense) and 5′-TCACAAGCACGTGCACTTTATTGAA-3′ (antisense), generating a 553-bp fragment of 9-27 cDNA; 5′-CATGAACCACATTGTGCAAACCT-3′ (sense) and 5′-GAAACATATACACTTTATTGAATG-3′ (antisense), generating a 626-bp 1-8D cDNA; and 5′-CATGAGTCACACTGTCCAAACCT-3′ (sense) and 5′-CCAGAAACACGTGCACTTTAT-3′ (antisense), generating a 563-bp 1-8U cDNA. The PCR fragments were blunt-ended with the Klenow fragment of the DNA polymerase and inserted into Sma I–digested pBluescript KSII (Stratagene). The cDNAs were ligated as Hind III–Bam HI fragments into the Hind III–Bam HI–digested pLdKpA eukaryotic expression vector [B. Mermer, B. K. Felber, M. Campbell, G. N. Pavlakis, *Nucleic Acids Res.* **18**, 2037 (1990)], resulting in pL927, pL18U, and pL18D. The expression vector contains the HIV-1 LTR promoter to +80, a polylinker, and the SV40 polyadenylation signal. HLtat cells were transfected with 5 μg of Gag-expressing plasmid; 1 μg of luciferase expression plasmid (pRSVluc) [J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987)]; and 0, 1, 5, and 10 μg of pL927. The total amount of DNA in the transfection mixture was adjusted to 17 μg with carrier DNA. One day later, the cells were harvested for protein or RNA analysis.
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The Cloning of PIG-A, a Component in the Early Step of GPI-Anchor Biosynthesis

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The glycosylphosphatidylinositol (GPI) anchor is a membrane attachment structure of many proteins and occurs in a wide variety of eukaryotes from yeasts to mammals. The structure of the core of the GPI anchor is conserved in protozoa and mammals and so is its biosynthetic pathway. A complementary DNA encoding a human protein termed PIG-A (phosphatidylinositol glycan–class A) was cloned. PIG-A was necessary for synthesis of *N*-acetylglucosaminyl-phosphatidylinositol, the very early intermediate in GPI-anchor biosynthesis.

More than a hundred eukaryotic cell surface proteins are anchored to the cell membrane by a GPI anchor (*1*). The GPI anchor acts not only as a membrane attachment structure but also as a sorting signal of apically expressed proteins of epithelial cells (*2*, *3*), in a signal transduction mechanism of leukocytes (*4*), as a target of phospholipases that release GPI-anchored proteins (*2*), and in a mechanism that confers increased lateral mobility of membrane proteins (*5*, *6*). Chemical and biosynthetic analyses of the GPI anchor have demonstrated that the core structure of the anchor is conserved in a variant surface glycoprotein of *Trypanosoma brucei* (*7*) and rat T lymphocyte protein Thy-1 (*8*) and that its biosynthetic pathways in protozoa and mammals are similar (*9*–*12*). The molecular cloning of genes encoding enzymes that take part in the biosynthesis of the GPI anchor is necessary to understand the biosynthetic pathway of the anchor and to understand the human disease paroxysmal nocturnal hemoglobinuria, which results from a deficiency of the GPI anchor (*13*).

To clone a cDNA encoding a protein that participates in GPI-anchor biosynthesis, we expressed cDNAs from a HeLa cell library with an Epstein-Barr virus vector (*14*) in the human B lymphoblastoid cell line JY-5, in which synthesis of the GPI anchor is deficient (*15*). Cell lines deficient in GPI-anchor synthesis have been grouped into several complementation classes (*16*), and their biochemical defects have been characterized (*10*–*12*). We analyzed the complementation class of the JY-5 cells by fusing them with murine Thy-1–deficient thymoma cell lines of known classes. JY-5 cells belong to class A that is deficient in an early step of GPI-anchor synthesis (*17*). Transfected JY-5 cells that expressed GPI-anchored proteins were selected by fluorescent flow cytometric sorting (*18*).

After three cycles of sorting and expansion by culture, we identified a cDNA clone

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that complemented deficient surface expression on JY-5 cells of the GPI-anchored proteins CD59 and decay-accelerating factor (DAF), both of which are complement regulatory proteins (Fig. 1) (*18*). We termed this gene PIG-A (phosphatidylinositol glycan–class A) and this cDNA clone pEBPIG-A. CD59 and DAF expressed on JY-5 cells transfected with pEBPIG-A were sensitive to phosphatidylinositol-specific phospholipase C (PI-PLC), which indicates that the GPI-anchor biosynthesis did occur (Fig. 1). This was confirmed by analysis of glycolipid biosynthesis (Fig. 2) (*19*). JY-5 cells did not synthesize *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI), the very early intermediate of GPI-anchor biosynthesis (9). This result is consistent with reports that the class A mutant is one of the three early mutants that do not synthesize GlcNAc-PI (*10, 20*). In cells transfected with pEBPIG-A, GlcNAc-PI was synthesized and subsequently deacetylated to form glucosaminyl-phosphatidylinositol (GlcN-PI), the second intermediate in GPI-anchor biosynthesis. Identification of these glycolipids was confirmed by their sensitivities to PI-PLC and nitrous acid (Fig. 2). Therefore, PIG-A participates in the very early step of GPI-anchor biosynthesis.

PIG-A cDNA consists of 3589 base pairs and codes for a predicted protein of 484 amino acids starting at the 86th base (Fig. 3). No similarity with PIG-A was found in DNA and protein databases. There is no apparent NH_2-terminal signal peptide sequence. Near the COOH-terminus is a hydrophobic sequence of 27 residues that may act as a transmembrane domain; this is followed by a 42-residue hydrophilic sequence (Fig. 3). Biosynthesis of the GPI anchor occurs in the endoplasmic reticulum (ER). Thus, PIG-A may be a membrane protein that resides in the ER with the NH_2-terminal portion in the cytoplasm. If the arginine immediately COOH-terminal to the putative transmembrane domain does not act to terminate translocation of the peptide across the ER membrane, the COOH-terminal domain might be in the lumen of the ER.

Expression of PIG-A cDNA in BW5147 class A cells that lack Thy-1 (Thy-1$^-$) (*21*) also complemented deficient surface expression of Thy-1 (Fig. 4) and deficient biosynthesis of GlcNAc-PI (*17*), which indicates that human PIG-A protein is compatible with the murine biosynthetic system of the GPI anchor and which further confirms that

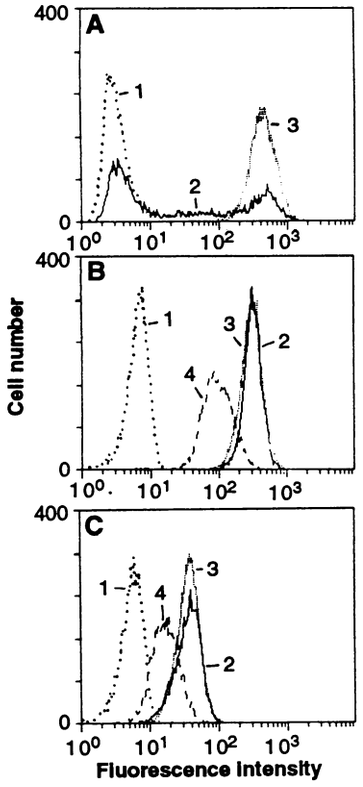

Fig. 1. Expression cloning of PIG-A cDNA. (**A**) Enrichment of CD59-positive cells by repeated sorting and growth of JY-5 cells after transfection of the cDNA library. Line 1, JY-5 cells before transfection; line 2, JY-5 cells after the third sorting (30% positive); line 3, wild-type JY-25 cells. Only 0.1% of the cells were positive after the second sorting. (**B** and **C**) Surface expression of GPI-anchored CD59 (B) and DAF (C) on JY-5 cells transfected with cloned PIG-A cDNA. The pEBPIG-A plasmid was transfected into JY-5 cells. After hygromycin selection, the cells were stained for CD59 (B) and DAF (C) before (line 2) and after (line 4) treatment with PI-PLC (Funakoshi, Tokyo). Line 1, untransfected JY-5 cells; line 3, wild-type JY-25 cells.

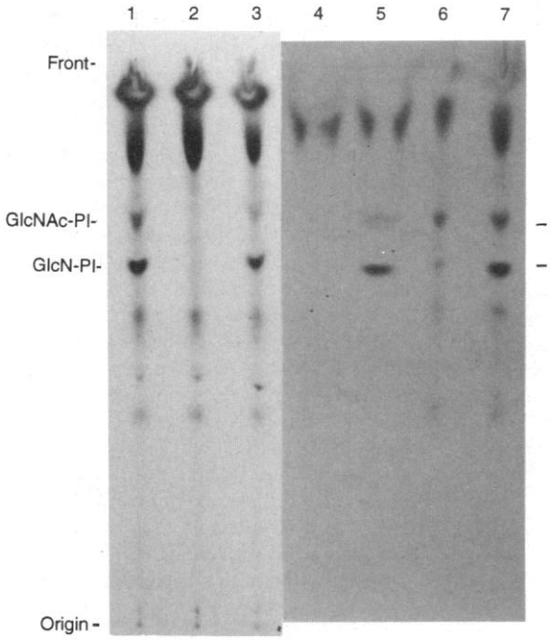

Fig. 2. Restoration of GlcNAc-PI synthesis in JY-5 cells with PIG-A cDNA. GlcNAc-PI and GlcN-PI were synthesized by a lysate of wild-type JY-25 cells (lane 1) (*19*). The glycolipids synthesized by the lysate of JY-25 cells were treated with PI-PLC (lane 4), enzyme buffer alone (lane 5), nitrous acid in acetate buffer (lane 6), or acetate buffer alone (lane 7) (*27*). JY-5 cells did not synthesize GlcNAc-PI and GlcN-PI (lane 2), but PIG-A–transfected JY-5 cells synthesized both glycolipids (lane 3).

A

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1          11         21         31         41         51
MACRGGAGNG HRASATLSRV SPGSLYTCRT RTENICMVSD FFYPNMGGVE SHIYQLSQCL

61         71         81         91         101        111
IERGEKVIIV TRAYGNRKGI RYLTSGLKVY YLPLKVMYNQ STATTLFESL PLLRYIFVRE

121        131        141        151        161        171
RVTIIESSSS FSAMAEDALF HAKTMGLQTV FTDESLFGFA DVSSVLTNKL LTVSLCDTNH

181        191        201        211        221        231
IICVSYTSKE NTVLRAALNP EIVSVIPNAV DPTDFTPDPF RRHDSITIVV VSRLVYRKGI

241        251        261        271        281        291
DLLSGIIPEL CQKYPDLNFI IGGEGPKRII LEEVRERYQL HDRVRLLGAL EHKDVRNVLV

301        311        321        331        341        351
QGHIFLNTSL TEAFCMAIVE AASCGLQVVS TRVGGIPEVL PENLIILCEP SVKSLCEGLE

361        371        381        391        401        411
KAIFQLKSGT LPAPENIHNI VKTFYTWRNV AERTEKVYDR VSVEAVLPMD KRLDRLISHC

421        431        441        451        461        471
GPVTGYIFAL LAVFNFLFLI FLRNMTPDSI IDVAIDATGP RGAWTNNYSH SKRGGENNEI

481
SETR
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B

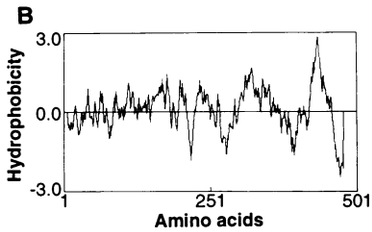

Fig. 3. (**A**) Deduced amino acid sequence of PIG-A cDNA (*28*). A 3.6 kb-insert of pEBPIG-A was subcloned into a pBluescript vector (Stratagene) in both directions, and nested deletion mutants were prepared. The nucleotide sequences of both strands were determined by the dideoxy termination method with a taq dye primer cycle sequencing kit and Model 370A DNA Sequencing System (Applied Biosystems). A stop codon is present 21 bp upstream of the predicted initiation codon. A putative hydrophobic transmembrane region is underlined. The accession number for the nucleotide sequence of PIG-A is D11466 (DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank). (**B**) Hydrophobicity plot of PIG-A.

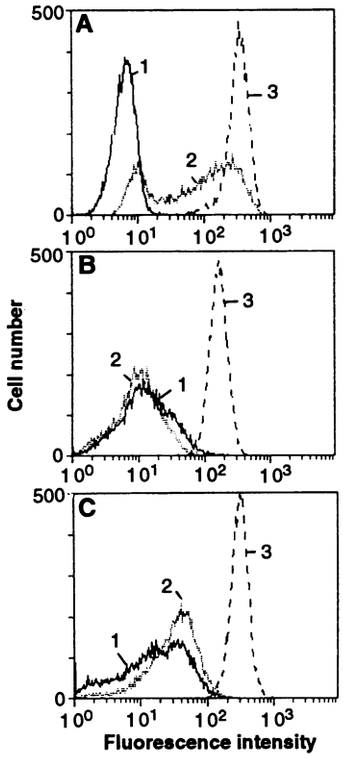

Fig. 4. Restoration of surface expression with PIG-A cDNA of Thy-1 murine class A mutants but not of C and H mutants. We introduced the neomycin resistance gene derived from pcD2 into the Sfi I site of pEBPIG-A by replacing a portion that contains oriP and the hygromycin resistance gene. The resulting PIG-A cDNA plasmid was transfected into Thy-1$^-$ murine thymoma cells (*29, 30*). Transfected cells were selected with G418, stained for Thy-1, and analyzed in a FACScan (Becton Dickinson). (**A**) BW5147 Thy-1$^-$ class A cells (line 1); PIG-A–transfected BW5147 Thy-1$^-$ class A cells (line 2); and wild-type BW5147 cells (line 3) stained for Thy-1.1. (**B**) TIMI Thy-1$^-$ class C cells (line 1); PIG-A–transfected TIMI Thy-1$^-$ class C cells (line 2); and wild-type EL4 cells (line 3) stained for Thy-1.2. (**C**) S49 Thy-1$^-$ class H cells (line 1); PIG-A–transfected S49 Thy-1$^-$ class H cells (line 2); and wild-type EL4 cells (line 3) stained for Thy-1.2.

PIG-A takes part in the early step of GPI-anchor biosynthesis. These results were confirmed with another class A cell line derived from S49 murine thymoma cells (*22*).

There are two other complementation classes, C and H, that are also early mutants, which indicates that three genes are required for biosynthesis of GlcNAc-PI (*10, 12, 20*). Because these three mutant cell lines show normal biosyntheses of phosphatidylinositol and uridine diphosphate (UDP)–GlcNAc, the three components seem to be necessary for the actual process of GlcNAc-PI synthesis. We expressed PIG-A in cells of classes C and H. PIG-A cDNA did not complement defects of these classes (Fig. 4).

Thus, PIG-A appears to function independently of the other components. The biosynthesis of GlcNAc-PI is thought to occur on the luminal side of the ER (*23*). If this is so, a transporter that transports UDP-GlcNAc from the cytoplasm to the lumen of the ER is required in addition to the enzyme GlcNAc transferase, which transfers GlcNAc from UDP-GlcNAc to PI. PIG-A has no sequence similarity with known GlcNAc transferases. Because sequence similarity is not always found among glycosyltransferases, a definitive conclusion on the function of the PIG-A protein is not possible. With Northern (RNA) blot analysis with a PIG-A cDNA probe, a single transcript of 4.2 kb was detected in wild-type JY cells. A faint signal was obtained around the 4.2 kb position with JY-5 cells (Fig. 5).

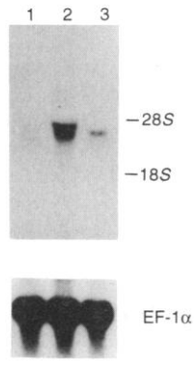

Fig. 5. Northern blot analysis of PIG-A transcripts in wild-type JY-25 and JY-5 cells. Samples of total RNA (10 μg each) (*31*) prepared from JY-25 cells (lane 3), JY-5 cells transfected with PIG-A (lane 2), and JY-5 cells (lane 1) were subjected to Northern blot analysis with a ^{32}P-labeled 2.7-kb Nco I fragment of PIG-A cDNA that contains the entire coding region and a part of the 3′ untranslated region as a probe. The blot was rehybridized with an elongation factor–1α (EF-1α) probe (*32*) to determine the amounts of RNA applied.

Our results show that PIG-A cDNA encodes a protein that functions in GPI-anchor biosynthesis. Our method of expression cloning may also be useful in cloning genes for other steps of GPI-anchor biosynthesis when appropriate mutant cells become available.

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18. A 5.6-kb Sfi I fragment containing oriP, a replication origin of Epstein-Barr virus, and a hygromycin resistance gene (but not the *EBNA-1* gene) was prepared from EBO-pSV2-neo (*14*) and was ligated to the phosphatase-treated Sfi I site of the pcD vector bearing cDNAs from a HeLa library that consisted of 3×10^6 clones (*24*). The library thus converted to the Epstein-Barr virus vector was introduced into *Escherichia coli* strain DH10B by electroporation, and plasmids were prepared. The converted library contained 1.7×10^7 independent clones, and 95% of the clones contained an insert with a mean size of 1 kb. Five portions of 10^8 JY-5 cells were transfected with 20 μg each of the library plasmids by electroporation at 960 μF and 250 V and cultured. After selection with hygromycin B, transfected cells (10^8 cells) were stained with a mixture of monoclonal antibody to CD59 and monoclonal antibody to DAF (*25*) and sorted with a fluorescence-activated cell sorter (FACStar, Becton Dickinson). The brightest 0.3% of the cells were collected and grown to 10^8 cells in culture. The staining, sorting, and expansion were done twice more. The plasmids were rescued from the selected JY-5 cells in *E. coli* by Hirt extraction and transformation (*26*). The plasmids were prepared from individual colonies, and inserts were analyzed. Twenty of 56 plasmids contained 3.6-kb inserts. Four of these plasmids were introduced into JY-5 cells. Three days later, all of them were found to have conferred surface expression of CD59 and DAF. One of them was designated as pEBPIG-A and retransfected into JY-5 cells.
19. We synthesized the GPI-anchor precursors in vitro by incubating cell lysates (from 10^7 cells) with 2 μCi of UDP-[6-^{3}H]-GlcNAc (American Radiolabeled Chemicals) at 37°C for 15 min (*27*). The lipid fraction was analyzed by silica gel thin-layer chromatography with solvent that consisted of a mixture of chloroform, methanol, and 1 M NH_4OH (10:10:3, by volume) (*27*), and glycolipids were detected by fluorography. To confirm that lipids contained phosphatidylinositol and glucosamine, we treated the lipid fraction with PI-PLC and HNO_2, respectively, before analysis (*27*).
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