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PIG-C, One of the Three Human Genes Involved in the First Step of Glycosylphosphatidylinositol Biosynthesis Is a Homologue of *Saccharomyces cerevisiae* GPI2

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Glycosylphosphatidylinositol (GPI) protein anchors are ubiquitous in eukaryotic cells. GPI anchors are synthesized in the endoplasmic reticulum by actions of ten or more gene products. The first step of the biosynthesis, the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to phosphatidylinositol, is mediated by at least three genes in mammalian cells (PIG-A, PIG-H and PIG-C) and in yeast (GPI1, GPI2 and GPI3/SPT14/CWH6). PIG-A is homologous to GPI3/SPT14/CWH6. However, PIG-H has no homology with GPI1 or GPI2. Here we cloned a human homologue of GPI2 and showed that it is PIG-C. PIG-C protein is a 297 amino-acid membrane protein in the endoplasmic reticulum that has 20 % amino acid identity with GPI2. Since there are several human EST sequences that have homology to GPI1, our results suggest that four genes are involved in the first step of GPI anchor synthesis in mammalian cells.

Glycosylphosphatidylinositol (GPI)-anchors are used by many eukaryotic membrane proteins for expression on the cell surface (reviewed in (1-4)). GPI-anchoring is a posttranslational modification occurring in the endoplasmic reticulum (ER). Precursors of the GPI-anchors are synthesized in the ER(5,6) and are transferred to nascent proteins en bloc by a transamidation reaction(7). The structure of the GPI anchor precursor is conserved among different organisms.

Ten or more genes are involved in the biosynthesis of GPI anchor precursors(5,6), which basically consists of sequential additions of sugars and ethanolaminephosphate to phosphatidylinositol (PI). The first step is the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI to generate the first intermediate GlcNAc-PI(8-11). This simple reaction is however, mediated by at least three genes, because there are three mutants in this step in both mammalian(10,12) and yeast(13) systems.

In the mammalian system, three complementation groups of mutants, class A, C and H, are defective at this step. PIG-A, a class A gene(14), and PIG-H, a class H gene(15), have been cloned but PIG-C, a class C gene, has not. The human PIG-A gene encodes a protein of 484 amino acids(14). It is an ER transmembrane protein with a large cytoplasmic domain and a small lumenal domain (Watanabe, R., submitted). The cytoplasmic domain has homology with the bacterial GlcNAc transferase, RfaK, that is involved in lipopolysaccharide synthesis(16), suggesting that PIG-A bears a catalytic site for GlcNAc transfer(17). Human PIG-H gene encodes a protein of 188 amino acids(15). It is also expressed in the ER (Watanabe, R., submitted). It has a hydrophobic region in the middle of the molecule but whether it is a transmembrane domain is not clear. A proteinase K protection assay has demonstrated that both the amino- and carboxy-termini face the cytoplasm (Watanabe, R., submitted). PIG-H

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has no significant homology with other proteins in the data bases, so its function cannot be predicted from its primary structure.

There are also three *Saccharomyces cerevisiae* mutants in the first step (gpi1, gpi2 and gpi3/spt14/cwh6)(13). All three genes have been cloned. GPI3/SPT14/CWH6(18,19)is homologous to mammalian PIG-A(17,19-21). It consists of 452 amino acids and has about 47 % amino acid identity with PIG-A(20). GPI1 encodes a protein of 60 kDa that has no homology to PIG-H(22). GPI2 encodes a protein of 281 amino acids that has also no homology to PIG-H(21). Both GPI1 and GPI2 proteins have multiple hydrophobic segments that may be transmembrane domains(21,22).

To further understand the relationships among the genes involved in the first step of GPI-anchor biosynthesis in yeast and mammalian systems, we identified a human homologue of GPI2 and showed that it is PIG-C.

MATERIALS AND METHODS

Identification of a human homologue of GPI2. We performed a tblastn search of an amino acid sequence of *S. cerevisiae* GPI2 in a human EST data base(23). EST R93920 (5′ sequence of clone 197133) gave the highest homology score. The insert size of this clone was 966 bp and the EST sequence was homologous to a region in the middle of GPI2 of about 1200 bp, indicating that 197133 is a partial clone. To find a full-length clone, we searched (blastn) a data base for the 3′ sequence of clone 197133 (EST R93921) and identified three ESTs, Z39278, R98011 and R01155. They were 3′ sequences of clones C-16b08, 201634 and 124854, respectively. Among them clone 201634 had the longest estimated insert of 1804 bp and its 5′ sequence (EST R98245) had homology to the 5′ end of GPI2. A search for homology with this 5′ sequence yielded three more ESTs, but they corresponded to downstream sequences. We purchased a plasmid of the I.M.A.G.E. Consortium Clone ID 201634(24) through Research Genetics, Inc. (Huntsville, AL) and sequenced it. The accession number of this nucleotide sequence is D85418.

Cell lines. The mouse thymoma class C mutant T1M1-Thy-1$^-$c and its parental line T1M1, a class A mutant S49-Thy-1$^-$a, and a class H mutant S49-Thy-1$^-$h(25) were gifts from Dr. R. Hyman (Salk Institute, San Diego, CA). The human B lymphoblastoid cell line JY5(26) was a gift from Dr. Moon L. Shin (University of Maryland, Baltimore, MD). They were cultured in DMEM supplemented with 10 % fetal calf serum.

Transfection of cDNA into mouse thymoma cells and analysis of the surface expression of Thy-1. An insert of clone 201634 was cloned into the mammalian expression vector pMEneo and transfected into class C mutant thymoma cells by electroporation (10^7 cells in 0.8 ml Hepes buffered saline, pH 7.05 at 280 V and 500 μF). PIG-A(14) and PIG-H(15) cDNAs were also transfected into class A, H and C mutant thymoma cells. Two days later, samples of the cells were stained for Thy-1 with the biotinylated anti-Thy-1 monoclonal antibody G7(27) and phycoerythrin-conjugated streptavidin (Biomeda), then analyzed in a FACScan cytometer (Becton Dickinson). The rest of the cells were further cultured in the presence of 300 μg/ml G418 to obtain stable transfectants.

Analysis of glycolipid biosynthesis. Parental T1M1 cells, class C mutant cells and its PIG-C transfected cells were cultured for three hours in the presence of 5 μg/ml tunicamycin to inhibit N-linked oligosaccharide synthesis. The cells were hypotonically lysed(11). The cell lysates (10^7 cell equivalents) were pelleted and resuspended in a buffer consisting of 50 mM Hepes (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml leupeptin, 5 mM $MnCl_2$, 0.5 mM DTT, 1 mM ATP and 0.2 μg/ml tunicamycin, then incubated with UDP-[6-^{3}H]GlcNAc (14 μCi/ml, American Radiolabeled Chemicals, MO) for 60 min at 37°C(11). The reaction was terminated with chloroform/methanol (1:1). Insoluble materials were removed by centrifugation and the solution was dried. The dried materials were extracted with n-butanol and glycolipids in the butanol phase were analyzed by thin-layer chromatography on Kieselgel 60 (Merck, Germany) with a solvent system of chloroform/methanol/water (10:10:3)(11) and visualization by image-analysis using a BAS1000 analyzer (Fuji Film, Tokyo, Japan).

Determination of the intracellular expression site of PIG-C. A plasmid was constructed so that PIG-C was tagged with glutathione-S-transferase (GST) at the amino-terminus(28) and expressed in class C mutant thymoma and JY5 cells. The cells (4×10^8) were disrupted by nitrogen cavitation and homogenization with a tight pestle Dounce homogenizer. The membrane fraction was obtained by differential centrifugation, then further fractionated by sucrose density gradient centrifugation(29). The fractions were characterized by measuring protein levels and the organella marker enzymes, alkalinephosphodiesterase I for the plasma membrane, α-mannosidase II for the Golgi and dolichol-phosphate-mannose synthase for the ER(29). The GST-tagged PIG-C (GST-PIG-C) in the fractions was dissolved with NP40, collected by glutathione-Sepharose and Western blotted against anti-GST antibodies. The blots were visualized by chemiluminescence reactions (DuPont NEN, Boston, MA).

RESULTS AND DISCUSSION

Cloning of a human homologue of yeast GPI2. We identified a cDNA clone that contained the most 5′ EST sequence that has homology to GPI2. Figure 1 shows the nucleotide and

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-293        AGTAAACTGAGTTGATTTGACTTTATCTCAGTACTACTCTTGACCTTTCACAA
-240  CTTTCGTAGGTTCACAGTCTCTCTTTTTCTAGGAACTTGGCTGTGTTGTCCTGCCTCAGA
-180  GACAAATTCATCTATTGTAGGCCTAGCCCCTGCCTTTGAAAACAAGGAAAGGTTGGTAGA
-120  ACATCAACACAGCATGGAATTTCCAGGGAGGTCTCATTTCAAAACTTCATAAAGAACAAG
 -60  AACCACCTGGACTTCTGTGAGGGCGATGATTAAACTGGCCTGAGTTTGAATGAAAGGATA
   1  ATGTATGCTCAACCTGTGACTAACACCAAGGAGGTCAAGTGGCAGAAGGTCTTGTATGAG
      M  Y  A  Q  P  V  T  N  T  K  E  V  K  W  Q  K  V  L  Y  E      20
  61  CGACAGCCCTTTCCTGATAACTATGTGGACCGGCGATTCCTGGAAGAGCTCCGGAAAAAC
      R  Q  P  F  P  D  N  Y  V  D  R  R  F  L  E  E  L  R  K  N      40
 121  ATCCATGCTCGGAAATACCAATATTGGGCTGTGGTATTTGAGTCCAGTGTGGTGATCCAG
      I  H  A  R  K  Y  Q  Y  W  A  V  V  F  E  S  S  V  V  I  Q      60
 181  CAGCTGTGCAGTGTTTGTGTTTTTGTGGTTATCTGGTGGTATATGGATGAGGGTCTTCTG
      Q  L  C  S  V  C  V  F  V  V  I  W  W  Y  M  D  E  G  L  L      80
 241  GCCCCCCATTGGCTTTTAGGGACTGGCCTGGCTTCTTCACTGATTGGGTATGTTTTGTTT
      A  P  H  W  L  L  G  T  G  L  A  S  S  L  I  G  Y  V  L  F     100
 301  GATCTCATTGATGGAGGTGAAGGGCGGAAGAAGAGTGGGCAGACCCGGTGGGCTGACCTG
      D  L  I  D  G  G  E  G  R  K  K  S  G  Q  T  R  W  A  D  L     120
 361  AAGAGTGCCCTAGTCTTCATTACTTTCACTTATGGGTTTTCACCAGTGCTGAAGACCCTT
      K  S  A  L  V  F  I  T  F  T  Y  G  F  S  P  V  L  K  T  L     140
 421  ACAGAGTCTGTCAGCACTGACACCATCTATGCCATGTCAGTCTTCATGCTGTTAGGCCAT
      T  E  S  V  S  T  D  T  I  Y  A  M  S  V  F  M  L  L  G  H     160
 481  CTCATCTTTTTTGACTATGGTGCCAATGCTGCCATTGTATCCAGCACACTATCCTTGAAC
      L  I  F  F  D  Y  G  A  N  A  A  I  V  S  S  T  L  S  L  N     180
 541  ATGGCCATCTTTGCTTCTGTATGCTTGGCATCACGTCTTCCCCGGTCCCTGCATGCCTTC
      M  A  I  F  A  S  V  C  L  A  S  R  L  P  R  S  L  H  A  F     200
 601  ATCATGGTGACATTTGCCATTCAGATTTTTGCCCTGTGGCCCATGTTGCAGAAGAAACTA
      I  M  V  T  F  A  I  Q  I  F  A  L  W  P  M  L  Q  K  K  L     220
 661  AAGGCATGTACTCCCCGGAGCTATGTGGGGGTCACACTGCTTTTTGCATTTTCAGCCGTG
      K  A  C  T  P  R  S  Y  V  G  V  T  L  L  F  A  F  S  A  V     240
 721  GGAGGCCTACTGTCCATTAGTGCTGTGGGAGCCGTACTCTTTGCCCTTCTGCTGATGTCT
      G  G  L  L  S  I  S  A  V  G  A  V  L  F  A  L  L  L  M  S     260
 781  ATCTCATGTCTGTGTCCATTCTACCTCATTCGCTTGCAGCTTTTTAAAGAAAACATTCAT
      I  S  C  L  C  P  F  Y  L  I  R  L  Q  L  F  K  E  N  I  H     280
 841  GGGCCTTGGGATGAAGCTGAAATCAAGGAAGACTTGTCCAGGTTCCTCAGTTAAATTAGG
      G  P  W  D  E  A  E  I  K  E  D  L  S  R  F  L  S              297
 901  ACATCCATTACATTATTAAAGCAAGCTGATAGATTAGCCTCCTAACTAGTATAGAACTTA
 961  AAGACAGAGTTCCATTCTGGAAGCAGCATGTCATTGTGGTAAGAGAATAGAGATCAAAAC
1021  CAAAAAAAATGAACCAAAGGCTTGGGTGGTGAGGGTGCTTATCCTTTCTGTTATTTTGTA
1081  GATGAAAAAACTTTCTGGGGACCTCTTGAATTACATGCTGTAACATATGAAGTGATGTGG
1141  TTTCTATTAAAAAAATAATACATCCAAAAAAAAAAAAAAAAAA
                          1165
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FIG. 1. Nucleotide and deduced amino acid sequence of PIG-C. Nucleotide numbers are on the left and amino acid numbers are on the right. A putative poly A signal(32) is double underlined.

deduced amino acid sequences of this human GPI2 homologue. It consisted of 1458 bp. All of eight EST sequences found in the dbEST by BLAST searches with GPI2 sequence were found in this sequence. The deduced human Gpi2p homologue had 297 amino acids that is 16 residues bigger than 281 amino-acid GPI2 protein. They had 20 % identity at the nucleotide level and 20 % identity (56 of 281) at the amino acid level (Fig. 2a). There was no hydrophobic amino-terminal signal sequence.

Identification of the GPI2 homologue as PIG-C. To determine if the human homologue of GPI2 corresponded to PIG-C, we cloned it into a mammalian expression vector and transfected it into class C mutant cells. A GPI2 homologue cDNA restored the surface expression of the GPI-anchored protein Thy-1 on the mutant in stable (Fig. 3a) and transient (data not shown) expression systems.

a

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PIG-C  MYAQPVTNTKEVKWQKVLYERQPFPDNYVDRRFLEELRKNIHARKYQYWAVVFESSVVIQ   60
GPI2   --------MTRSPWKRLLWLKQEYPDNYTDPSFIE-LRARQKAESNQKSDRKLSEARRAQ   51
                *...*  .* .**** *  *.* **   .*   *        .   *

PIG-C  QLCSVCVFVVIWWYMDEGLLAPHWLLGTGLASSLIGYVLFDLIDGGEGRKKSG--QTRWA  118
GPI2   IRLDFISFYQTILNTSFIYITFTYIYYYGFDP-IPPTIFLSFITLIISRTKVDPLLSSFM  110
          *           ..   .   *    .   .    *     * *     .

PIG-C  DLKSALVFITFTYGFSPVLKTLTESVSTDTIYAMSVFMLLGHLIFFDYGANAAIVSSTLS  178
GPI2   DVKSSLIITFAMLTLSPVLKSLSKTTASDSIWTLSFWLTLWYIFVISSTK-SKDKPSNLS  169
       *.**.*.        *****.*. . ..*.* ..*  . *  .         .    *.**

PIG-C  LNMAIFASVCLASRLPRSLHAFIMVTFAIQIFALWPMLQKKLKACTPRSYVGVTLLFAFS  238
GPI2   TNILVALVAVLSSRLSTTIDVFCFLLICIQLNIILPTYLSVTNKVVPIISNIIVYSFLNV  229
        *. .     *.***  ..  *  .  .**.  . *          *     .   *

PIG-C  AVGGLLSISAVGAVLFALLLMSISCLCPFYLIRLQLFKENIHGPWDEAEIKEDLSRFLS   297
GPI2   ALGWIYMLLIFFASVFYITVLPK--WFIYWKINYHKRDNDLLSTWDARTPILDZ-----   281
       *.* .  .    * .* . ..       .  *  .     .   **      *
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b

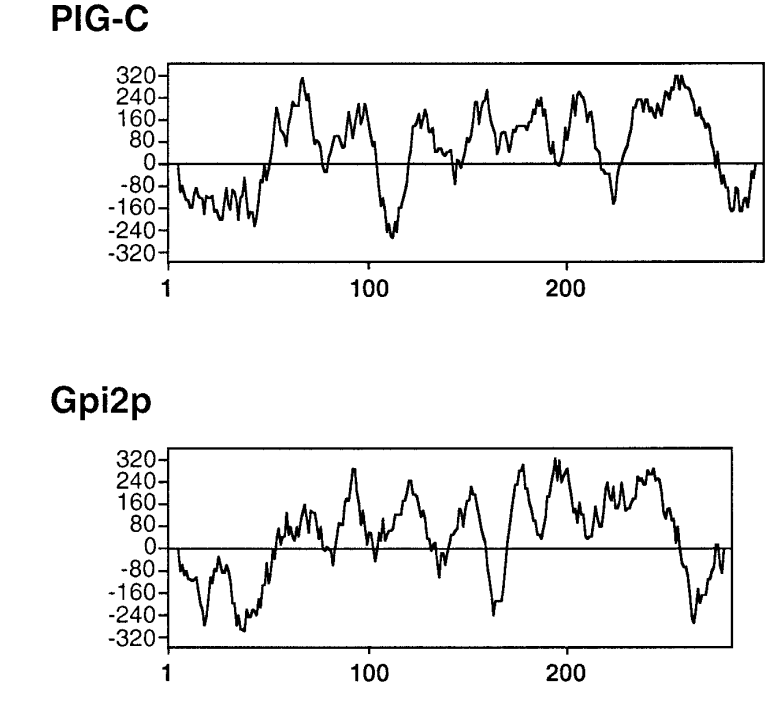

FIG. 2. Comparison of PIG-C with GPI2. (a) Comparison of the amino acid sequences of PIG-C and GPI2. Sequences were aligned using the Clustal W program. Asterisks and dots indicate identical and similar amino acid residues, respectively. (b) Hydropathy profiles of PIG-C and GPI2 drawn by the Kyte and Doolittle program.

We assessed the restoration of glycolipid synthesis. Class C cells are defective in the first step of GPI anchor biosynthesis, so the first intermediate, GlcNAc-PI is not synthesized (Fig. 3b, lane 1). After transfection with a cDNA of the human GPI2 homologue, the first and

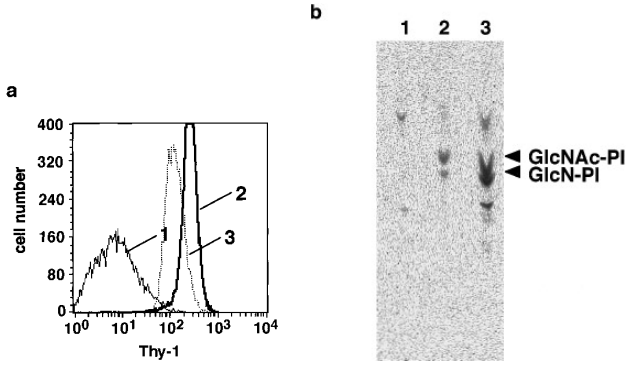

FIG. 3. Complementation of class C mutant with PIG-C cDNA. (a) Surface expression of Thy-1. Class C mutant cells (line 1), its PIG-C-transfectants (line 2) and parental T1M1 cells (line 3) were stained for Thy-1. (b) Glycolipid biosynthesis. Lysates of class C mutant cells (lane 1), parental T1M1 cells (lane 2) and PIG-C-transfected class C mutant cells (lane 3) were incubated with UDP-[^{3}H]GlcNAc, then glycolipids were analyzed by thin-layer chromatography and fluorography. Spots of the first (GlcNAc-PI) and the second (glucosaminyl-PI; GlcN-PI) intermediates are marked on the right.

second intermediates were biosynthesized (Fig. 3b, lane 3). Therefore, biosynthesis of the GPI anchor and subsequent surface expression of GPI-anchored proteins were restored in class C mutants by the GPI2 homologue. Thus, we identified GPI2 homologue as PIG-C.

PIG-C is an ER membrane protein. A hydropathy profile of PIG-C showed seven hydrophobic segments (Fig. 2b). Yeast Gpi2p also has seven hydrophobic segments (Fig. 2b), which may be transmembrane domains. Since GPI anchors are synthesized in the ER, this hydropathy profile of PIG-C suggests that PIG-C is an ER membrane protein with several transmembrane domains.

To localize the subcellular expression site of PIG-C, we tagged PIG-C with GST and expressed it in class C and JY5 cells. GST-tagged PIG-C complemented the class C mutant, indicating that the GST-PIG-C fusion protein functioned as PIG-C (data not shown). Western blotting analysis of the subcellular fractions from JY5 cells with anti-GST antibody demonstrated that GST-PIG-C (about 50 kDa) was primarily expressed in the fractions containing the ER marker (fractions 3, 4 and 5, Fig. 4). Another band at 100 kDa position may be dimerized GST-PIG-C or GST-PIG-C interacting with something else, presumably due to its hydrophobic nature, because with lower concentrations of SDS in the sample buffer this band increased with a concomitant loss of the 50 kDa band of GST-PIG-C (data not shown).

Relationships of PIG-C with PIG-A and PIG-H. PIG-C cDNA did not complement two other mutants, class A and H, that are also defective in the first sep (Fig. 5a and b). Likewise, PIG-H cDNA (Fig. 5c) and PIG-A cDNA(14) did not complement the class C mutant. Thus, PIG-C does not function as an upstream or a downstream factor of PIG-A or PIG-H. Both PIG-A and PIG-H are ER membrane proteins. Since PIG-C is also an ER membrane protein (Fig. 4), all three proteins may directly participate in the first reaction in the ER as a catalytic or regulatory subunit.

Comparison of the genes involved in the first step of GPI-anchor synthesis in mammalian and yeast systems. The results of this and other studies(17,20,21) demonstrated that mammalian PIG-A and PIG-C are homologous to yeast GPI3 and GPI2, respectively. In contrast, PIG-H is not structurally homologous to GPI1(22). BLAST searches of the whole genome sequence of *S. cerevisiae* with the PIG-H sequence did not identify any significantly homologous sequences. Therefore, there is no PIG-H homologue in yeast or it is so weakly homologous to PIG-H that regular BLAST searches did not detect it. On the other hand, there are human EST

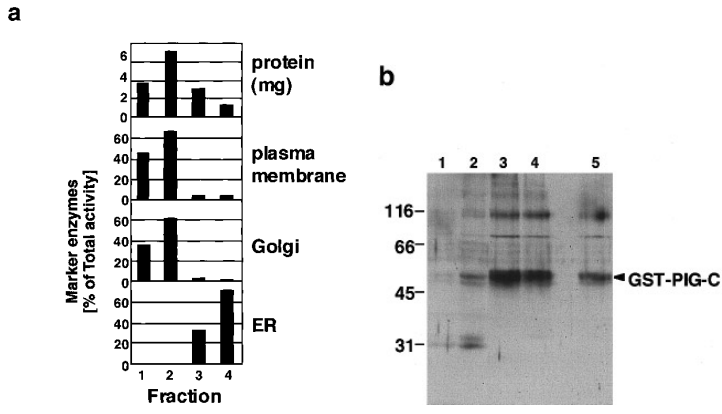

FIG. 4. Expression of PIG-C in the ER demonstrated by Western blotting after subcellular fractionation. (a) Distribution of protein and organella marker enzymes in fractions 1-4 after sucrose density gradient centrifugation. (b) Detection of GST-PIG-C by Western blotting with anti-GST antibody. Lanes 1-4, fractions 1-4 respectively after sucrose density gradient centrifugation as in (a). Lane 5, pellet after sucrose density gradient centrifugation. Molecular size markers (kDa) are on the left.

sequences that have homology to GPI1(22). Although the function of this human GPI1 homologue has to be determined, our results suggested that four genes may be involved in the first step of GPI anchor synthesis. The molecular basis as to why so many proteins are involved in this GlcNAc transfer remains to be clarified.

The ESTs corresponding to PIG-C have been grouped into a cluster in the UniGENE

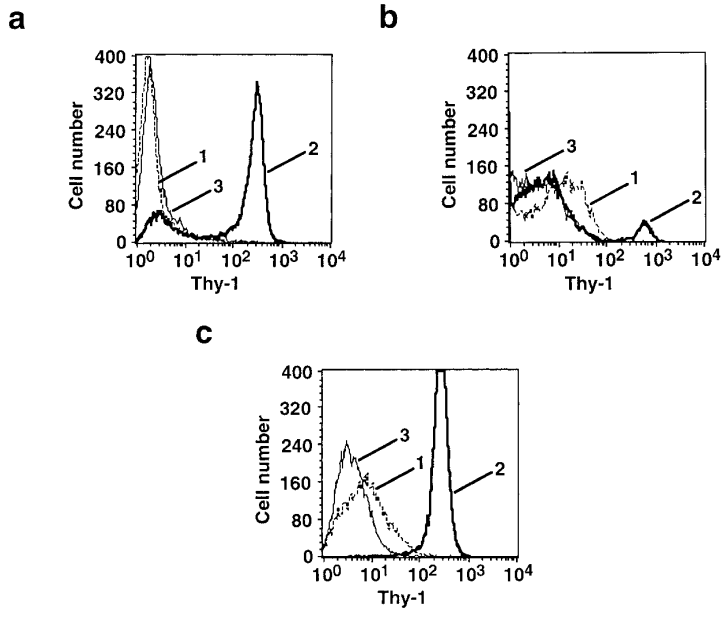

FIG. 5. Lack of cross-complementation between three genes and mutants for the first reaction step. (a) Class A mutant cells transfected with PIG-A cDNA (line 2) and PIG-C cDNA (line 3). Line 1 represents untransfected class A cells. (b) Class H mutant cells transfected with PIG-H cDNA (line 2) and PIG-C cDNA (line 3). Line 1 represents untransfected class H cells. (c) Class C mutant cells transfected with PIG-C cDNA (line 2) and PIG-H cDNA (line 3). Line 1 represents untransfected class C cells.

sets(30). This cluster has been mapped to chromosome 1. This autosomal location of PIG-C should account for why it is not involved in the GPI-anchor deficiency, paroxysmal nocturnal hemoglobinuria which is always caused by somatic mutation of the X-linked gene PIG-A(31).

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