Cloning of a Human Gene, PIG-F, a Component of Glycosylphosphatidylinositol Anchor Biosynthesis, by a Novel Expression Cloning Strategy*

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The glycosylphosphatidylinositol (GPI)-anchored proteins are widely distributed in eukaryotic cells, from yeasts to mammals. A number of proteins, such as glycosyltransferases, are necessary for GPI anchor biosynthesis. Cloning of genes encoding these proteins is required for analyses of their nature and the biosynthetic pathway. Here we report a new method of expression cloning that is applicable to many mutant rodent and human cells, and its application in cloning a human cDNA termed PIG-F (for Phosphatidylinositol-Glycan class F) using a Thy-1-negative mutant murine thymoma cell line of complementation class F. PIG-F takes a part in the step of transfer of ethanolamine phosphate to the GPI intermediate containing three residues of mannose. This expression cloning strategy is applicable to the identification of not only other genes involved in GPI anchor biosynthesis but also human disease-associated genes using mutant mammalian cell lines.

A number of eukaryotic cell surface proteins are anchored to the membrane by glycosylphosphatidylinositol (GPI),¹ the core structure of which is conserved in protozoan and mammalian cells (1, 2). Many mutants that are deficient in GPI anchor biosynthesis have been established from Chinese hamster ovary (CHO) cells (3), murine T-cell lymphoma cells (4), human lymphoblastoid JY cells (5), and human erythroleukemia K562 cells (6), and they have been used to analyze the GPI-anchoring pathway. They have been classified into several complementation classes by somatic cell fusion analysis (4), some of their biochemical defects being clarified by biosynthetic analyses (6-10). Little is known, however, about genes encoding proteins involved in biosynthesis of the GPI anchor, because it has been difficult to refine the proteins and to clone the genes using the mutant cell lines.

Expression cloning of cDNA is a very useful method, especially when the protein or DNA sequence is not available. In expression cloning, COS cells are widely used as recipient cells, because their constitutive expression of SV40 large T antigen (SV40T) allows replication of plasmids containing the SV40 origin and, hence, rescue of a particular plasmid from cells sorted based on their expression of a functional or antigenic marker (11, 12). This system, however, is not directly applicable for expression cloning of a cDNA involving complementation of a deficient phenotype of mutant cells other than COS cells. In such cases, the mutant cells must be converted to COS-like cells expressing a high level of SV40T or WOP-like cells (13) expressing a high level of polyoma virus large T antigen (PyT) to allow amplification of plasmids containing SV40 or polyoma virus origin of replication (Pyori), respectively (14, 15). For mutant adherent cells, expression of PyT is required, but making PyT-expressing mutant cells is time-consuming. Therefore cotransfection of a cDNA library containing Pyori in a vector with a plasmid encoding PyT into murine mutant cells is a useful method for cloning the genes of known functions but unknown primary structure.

In this study, we have successfully utilized this cloning method to clone PIG-F (phosphatidylinositol-glycan class F) cDNA, which induces Thy-1 expression on the cell surface following complementation of deficient GPI anchor biosynthesis in the Thy-1-negative mutant murine thymoma cell line of complementation class F (Thy-1‘ cell).

MATERIALS AND METHODS

Cell Lines and Culture—The murine thymoma cell line EL4, its Thy-1-negative mutant cell line (Thy-1‘ cell) (16), the human hepatoma cell line Hep3B, and the human erythroleukemia cell line K562 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. CHO cells and NIH3T3 cells were cultured in Ham's F-12 containing 10% fetal calf serum and Dulbecco's modified Eagle's medium containing 10% calf serum, respectively.

Construction of Plasmids—A 3.6-kbp BamHI fragment containing the PyT gene derived from the plasmid Mo/Mo gpt (17) and a 3-kbp BamHI fragment of the SV40T gene-containing SFFV-lacZ plasmid (18) were subcloned into the BamHI site of pBluescript II (pBS) [(Stratagene)] to obtain pBSPT and pBS-SV40T, respectively. The PyT gene fragment was excised from pBSPT with Smal and NotI and was inserted between the NruI and NotI sites of pcDNAI (Intrigen) to obtain pCDNAPT.

Chloroform Phosphatidyl Erythrosine Phosphatidyl Erythrosine Transaminase (CAT) Assay—The CAT gene was cloned into the BamHI site of pCEV4 vector (19). This plasmid, pCEVCAT, was cotransfected with pBSPT or pBS into 1 X 10⁶ Thy-1‘ cells, 3 X 10⁶ CHO cells, or 3 X 10⁵ NIH3T3 cells by electroporation under optimum conditions for each cell type: 350 V and 250 μF for Thy-1‘ cells, 300 V and 500 μF for CHO cells, and 250 V and 500 μF for NIH3T3 cells. pCEVCAT and pBS or pBSV40T were electroporated into 3 X 10⁶ Hep3B cells at 250 V and 250 μF, and into 1 X 10⁵ K562 cells at 250 V and 860 μF. After culture for 2 days, CAT

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‡ The abbreviations used are: GPI, glycosylphosphatidylinositol; CHO, Chinese hamster ovary; SV40T, simian virus 40 large T antigen; PyT, polyoma virus large T antigen; Pyori, polyoma virus origin of replication; PIG-F, phosphatidylinositol-glycan class F; Thy-1‘ cell, Thy-1-negative mutant murine thymoma cell line of complementation class F; pBS, pBluescript II; CAT, chloroform phosphatidyl acetyltransferase; PI, phosphatidylinositol; EthN-P, ethanolamine phosphate; PNH, paroxysmal nocturnal hemoglobinuria; PIG-A, phosphatidylinositol-glycan class A; μF, microfarads; kbp, kilobase pair(s).
activity was measured as described (20).

Expression Cloning System—An oligo(dT)-primed cDNA library consisting of 4.3 \times 10^6 clones and a random hexamer-primed cDNA library consisting of 4.0 \times 10^6 clones, both from the human T cell line KT-3, prepared in the vector pCEV4 (a gift from Dr. N. Itoh) were mixed (21). A sample of 5.0 \times 10^6 Thy-1' T cells in the logarithmic growth phase was mixed with 150 \mu g of the cDNA library plasmids and 150 \mu g of the pDNAPyT plasmids in 4.0 ml of Hepes-buffered saline buffer, consisting of 20 mM Hepes, pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose (22), and the mixture was introduced into five cuvettes of 0.4-cm width and subjected to electroporation at 350 V and 250 \mu F with a Gene Pulser (Bio-Rad). After culture for 2 days, the transfected cells were stained with biotinylated anti-Thy-1.2 monoclonal antibody G7 (a gift from Dr. T. Tadakuma) and phycoerythrin-conjugated streptavidin (Biomeda). The 110 brightest cells were collected by sorting in a FACStar cell sorter (Becton Dickinson). The plasmids were recovered from the collected cells by the Hirt method (23) and were transformed into Escherichia coli DH10B by electroporation. Colonies containing pCEV4-cDNA plasmids were selected on ampicillin plates. Then 480 of the 1100 colonies obtained were cultured individually in five 96-well plates (Corning). Five miniprep plasmid pools obtained from each plate were cotransfected into Thy-1' T cells with pBSpyT, and the cells were screened for Thy-1' expression. Three pools gave Thy-1'-positive signals on analysis by a FACScan (Becton Dickinson). The pool that showed the highest percentage of Thy-1'-positive cells was further divided into 20 pools, each corresponding to one of the 12 rows and 8 columns of the 96-well plate and rescreened again. Four positive clones were obtained on the final screening of individual wells from positive pools.

Colony Hybridization—Colony hybridization was carried out as described (20). The probe used was a 1-kbp XhoI fragment of the pCEVPIG-F-1 labeled with ^32P by the random primer labeling method.

Establishment of Transfectants Expressing PIG-F—The SfiI fragment of the neomycin resistance gene derived from pcD2 (a gift from H. Nojima) was inserted into the SfiI site of pCEVPPIG-F-1. This plasmid, pCEVneoPIG-F, was transfected into Thy-1' T cells by electroporation at 350 V and 250 \mu F. The next day, transfectedants were subjected to selection in medium with 400 \mu g/ml G418 (GIBCO). After 10 days, cloning of Thy-1'-positive transfectants was done by limiting dilution.

Nucleotide Sequence of PIG-F—A 1-kbp BamHI and PstI fragment of pCEVPIG-F containing the PIG-F gene was subcloned into the pBS plasmid, and deletion mutants were prepared by the exonuclease III method. The nucleotide sequences of both strands of pBS-PIG-F were determined by the dideoxy termination method with a Taq Dye Primer Cycle sequencing kit and an Applied Biosystems model 370A DNA sequencing system.

Bioactivity of GPI Anchor Intermediates—In vivo labeling of cells with [H]mannose and thin layer chromatographic analysis were done as described (24).

RESULTS

To determine whether cotransfection of a plasmid bearing PyT with a plasmid bearing Pyori would result in replication of the latter in the murine thymoma mutant cell, we transfected a plasmid bearing Pyori and the CAT gene (pCEVCAT) with a plasmid bearing PyT (pBSpyT) into the Thy-1' T cells and measured the expression of CAT activity. High CAT activity was detected in the cells transfected with pCEVCAT and pBSpyT (5100 dpm), but not with pCEVCAT and the vector pBS (1100 dpm; approximately the background level), suggesting that on cotransfection Py' supported replication of the PyT plasmid bearing Pyori.

We then prepared another plasmid bearing PyT with a pcDNA1 vector (pcDNAPyT) for use in expression cloning. This pcDNA1 vector does not contain the ß-lactamase gene, so this plasmid would not be rescued together with plasmids derived from a cDNA library if ampicillin were used in plasmid rescue. The procedure for expression cloning using Thy-1' T cells is described in Fig. 1.

A preliminary experiment showed that rearranged plasmids containing both the ß-lactamase gene and PyT gene arose after two cycles of sorting with a FACStar. Therefore, sorting was done only once to prevent rearrangement of plasmids.

As described under "Materials and Methods," four clones (termed pCEVPPIG-F-1 to -4) were obtained. These clones gave identical maps, suggesting that they were derived from a single clone. Using a 1-kbp insert of pCEVPPIG-F-1 as a probe, we screened the original oligo(dT)-primed KT-3 cDNA library and isolated two additional clones. One clone gave an identical map to pCEVPPIG-F-1, showing that pCEVPPIG-F-1 had not undergone any rearrangement during the cloning procedure. The other clone, pCEVPPIG-F-5, had a larger insert (2.0 kbp). Further analysis revealed that this large insert was a product of rearrangement between the 1-kbp cDNA from pCEVPPIG-F-1 and an unknown sequence.

For evaluation of the function of PIG-F cDNA, a neomycin resistance gene was inserted into pCEVPPIG-F-1 and the construct was transfected into Thy-1' T cells to establish a permanent cell line. After selection with G418, the transfected clearly expressed Thy-1.2 glycoproteins on the cell surface (Fig. 2a), suggesting that the PIG-F cDNA complemented the deficient GPI biosynthesis. For further confirmation of GPI anchor biosynthesis, we analyzed the mannolipids in parental wild-type cells (EL4), Thy-1' T cells, and cloned PIG-F-transfected Thy-1' T cells metabolically labeled with [H]mannose. As shown in Fig. 2b, mature type GPI anchors (marked C/H8 and H7) were clearly seen in the lysates of both the PIG-F transfectant and the wild-type cell (EL4), whereas in agreement with previous reports the M3 product, which is an immediate precursor of the mature types, was accumulated in the lysate of Thy-1' T cells (8). The wild-type EL4 and the PIG-F transfectant incorporated comparable amounts of [H]mannose into the mature types of GPI anchor. Ratio of H7 to C/H8 was apparently different in these two cells, but a reason for this is not known. These results together indicate that PIG-F is a factor involved in the late step of GPI anchor biosynthesis, i.e. the addition of ethanolamine phosphate (EthN-P) to the third mannose.

pCEVPPIG-F-1 has a 1.0-kbp insert, which contains a long

\[ N. \text{ Inoue, unpublished observations.} \]
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**Fig. 2.** Complementation of biochemical defect of Thy-1-f cells with PIG-F cDNA. a, complementation of surface expression of Thy-1. Wild-type EL4 (line 1), Thy-1-f cells (line 2), and PIG-F-transfected Thy-1-f cells (line 3). b, complementation of GPI anchor biosynthesis analyzed by incorporation of ['H]mannose and thin layer chromatography. Wild-type EL4 (lane 1), Thy-1-f cells (lane 2), and cloned PIG-F-transfected Thy-1-f cells (lane 3). M, to M, represent GPI precursors containing one to three mannose residues, respectively (8). C and DPM represent the complete GPI core and dolichol-phosphate-mannose, respectively (8). H5 to H8 represent GPI intermediates according to the terminology reported by S. Hirose et al. (24). The structure of H7 is proposed to be EthN-P-ManMan(EthN-P+)ManGlcN-PI (24). The structure of H8 has not been clearly determined. However, it has been suggested that EthN-P might be incorporated at the 6-position of the second Man in H7 (24).

open reading frame encoding a polypeptide of 219 amino acid residues (Fig. 3a). A typical consensus sequence for initiation of translation is found in the 5'-untranslated region (25). There is no typical N-terminal hydrophobic sequence of the signal peptide. No significant homology with PIG-F was found in DNA and protein data bases, indicating that PIG-F is a new protein. As shown in Fig. 3b, the deduced amino acid sequence of PIG-F indicates that the protein is very hydrophobic (more than 55%), suggesting that most of it is embedded in the membrane.

**DISCUSSION**

In this study we have successfully cloned PIG-F cDNA involved in the late step in GPI anchor biosynthesis. The involvement of PIG-F protein in GPI anchor biosynthesis was indicated by two criteria, namely Thy-1 expression on the cell surface (Fig. 2a) and biosynthesis of complete GPI anchor (Fig. 2b), which were induced by transfection of PIG-F cDNA into Thy-1-f cells. Because cloned cDNA does not have any DNA binding motifs, PIG-F protein may be directly involved in the process of GPI anchor biosynthesis. Previous biochemical analyses of Thy-1-f cells indicated that this mutant has a defect in the step of transfer of EthN-P from its donor phosphatidylethanolamine (26) to the GPI intermediate containing three residues of mannose (8, 24). Therefore, PIG-F may be an EthN-P transerase. Direct demonstration of transfer of EthN-P by the expressed PIG-F protein is required for confirmation of this possibility.

Recently Thy-1-f cells were reported to have a defect in ether lipid biosynthesis (Fig. 3a). A typical consensus sequence for initiation of translation is found in the 5'-untranslated region (25). There is no typical N-terminal hydrophobic sequence of the signal peptide. No significant homology with PIG-F was found in DNA and protein data bases, indicating that PIG-F is a new protein. As shown in Fig. 3b, the deduced amino acid sequence of PIG-F indicates that the protein is very hydrophobic (more than 55%), suggesting that most of it is embedded in the membrane.

**Fig. 3.** a, nucleotide and deduced amino acid sequence of PIG-F cDNA. The 917-base pair sequence of PIG-F is shown along with the deduced 219-amino acid sequence of the predicted translation product. Nucleotide numbering is shown above the sequence. A putative polyadenylation signal is boxed. b, hydrophobicity plot of the amino acid sequence of PIG-F according to the method of Kyte and Doolittle (31).

nation is that Thy-1-f cells are a double mutant harboring two independent mutations, one involved in alkylated PI biosynthesis and the other involved in the EthN-P transfer. In this case PIG-F may be involved in the late step of GPI anchor biosynthesis, as discussed above. These possibilities can now be examined experimentally by analyzing whether PIG-F-transfected Thy-1-f cells synthesize alkylated PI.

A deficiency of GPI anchor biosynthesis in human hematopoietic cells causes a hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH) (28, 29). Our recent investigations demonstrated that a defect of a gene termed PIG-A (for Phosphatidyl-Inositol-Glycan class A), which is involved in the early step of GPI anchor biosynthesis (30), is a cause of PNH. A defect at any step of GPI anchor biosynthesis could...
conceivably result in PNH, so it is not surprising that a few cases of PNH might involve a defect of PIG-F rather than of PIG-A.

The novel expression cloning system reported in this paper has a number of unique characteristics. A major feature is that it allows direct use of mutant rodent cells for expression cloning. To accomplish direct cloning using mutant cells, we cotransfected a cDNA library prepared with Pyori-containing vector and a plasmid encoding PyT into Thy-1-f cells. Feasibility of this cloning strategy was initially tested by cotransfecting pCEVCAT containing Pyori and pBSPyT to assess replication of the former. High CAT activity was detected only when pCEVCAT was cotransfected into the cells with pBSPyT, suggesting that PyT is able to increase the copy number of plasmids containing Pyori. The usefulness of this cotransfection method was confirmed using CHO cells and murine NIH3T3 cells. Thus this system should be applicable for cDNA cloning using a wide variety of mutant rodent cells, including other complementation classes of Thy-1-negative lymphoma cells. This protocol should also be applicable to human mutant cells by cotransflecting a cDNA library containing the SV40 origin of replication with a plasmid encoding SV40T, which was also shown with Hep3B cells and K562 cells.

Rescue of amplified cDNA plasmids from Thy-1-f cells was another important step in cDNA cloning. As a plasmid encoding PyT was cotransfected into Thy-1-f cells, pcDNAPyT, which does not contain the β-lactamase gene, was constructed to rescue only cDNA library-derived plasmids on ampicillin plates. Although after the first sorting plasmid selection on ampicillin plates. Although after the first sorting plasmid selection on ampicillin plates was effective, rearranged plasmids containing both the β-lactamase gene and the PyT gene appeared after two cycles of sorting, and rearranged plasmid had overcome cDNA plasmids after three cycles of sorting. Therefore, in our protocol, cell sorting was used only once, and plasmids collected from signal-positive cells were assayed by cotransfection with pBSPyT into Thy-1-f cells until a single clone was obtained.

Many kinds of mutant cell lines have been established to analyze functions of proteins of unknown primary structure and to clone genes of these proteins. However, it was difficult to clone the genes based on the phenotypic correction of the deficient mutant cells. This expression cloning strategy could facilitate the identification of these genes using mutant mammalian cell lines.

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REFERENCES