Cloning of murine glycosyl phosphatidylinositol anchor attachment protein, GPAA1

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Hiroi, Y., R. Chen, H. Sawa, T. Hosoda, S. Kudoh, Y. Kobayashi, H. Aburatani, K. Nagashima, R. Nagai, Y. Yazaki, M. E. Medof, and I. Komuro. Cloning of murine glycosyl phosphatidylinositol anchor attachment protein, GPAA1. Am J Physiol Cell Physiol 279: C205–C212, 2000.—Glycosyl phosphatidylinositol (GPI) anchor attachment protein, GPAA1, is about 2 kb in length and encodes a putative 621 amino acid protein. The mGPAA1 gene has 12 small exons and 11 small introns. mGPAA1 mRNA is ubiquitously expressed in mammalian cells, and in situ hybridization analysis revealed that it is abundant in the choroid plexus, skeletal muscle, osteoblasts of rib, and occipital bone in mouse embryos. Its expression levels and transamidation efficiency decreased with differentiation of embryonic stem cells. The 3T3 cell lines failed to express GPI-anchored proteins have been identified (3, 17, 18). Among these mutants, the two affected genes, GAA1 and GPI8, have been isolated and characterized. GAA1 and GPI8 are yeast mutants that can synthesize the complete GPI precursor but fail to express GPI-anchored proteins on the cell surface (2, 8). Their phenotypes are similar to that of the mammalian class K mutant for which a defect in the activity of the transamidase has been documented (4, 24). The affected gene in mutant K cells, the PIG-K gene, recently has been shown to be hGPI8 (human GPI8), a homologue of yGPI8 (yeast GPI8) (37).

In this study, we isolated murine GPAA1 cDNA and the murine GPAA1 gene, examined its expression, and characterized GPAA1 protein function.

METHODS

The signal sequence trap method and murine cDNA library screening. The signal sequence method was performed as described (10, 32). We used poly(A)− RNA from embryoid bodies (EB) that had been differentiated for six days. We screened ~1.0 × 108 plaques of a murine heart cDNA library in Uni-ZAP XR Vector (Stratagene) with a 32P-labeled cDNA fragment of clone 82D3 that appeared to be a new gene.
Hybridization was performed at 42°C for 12 h in 2× sodium chloride-sodium citrate (SSC), 50% deionized formamide, 10% dextran sulfate, 1% SDS, and 20 mg/ml heat-denatured salmon sperm DNA. The filters were washed twice with 2× SSC/0.1% SDS, once with 1× SSC/0.1% SDS, and twice with 0.1× SSC/0.1% SDS at 65°C. Plasmids were excised from the phage vectors in vivo by the ExAssist helper phage (Stratagene). Nucleotide sequences of clones were determined on both strands using the fluorescent dideoxy terminator sequencing kit (Perkin-Elmer).

**Murine genomic library screening.** Approximately 1.0 × 10⁶ plaques of a murine 129SV genomic library in λFIXII Vector (Clontech) were screened with ³²P-labeled mGPAA1 (murine GPI anchor attachment) cDNA. Phage DNA was purified from the isolated clone, digested with EcoR-I, and subcloned into the EcoRI site of pBluescript II SK(+). This plasmid was digested with several restriction enzymes to produce a restriction enzyme map. About 8.9 kb of nucleotide sequence was determined on both strands.

**Northern blot analysis.** Total RNA was extracted from murine fetal, neonatal, and adult tissues with RNAzol B (Biotex Laboratories). Twenty micrograms of total RNA were separated on 1.0% formaldehyde-agarose gel and transferred to Hybond-N filters (Amersham). The filters were
hybridized with 32P-labeled mGPAA1 cDNA at 42°C for 12 h in 5× sodium chloride-sodium phosphate-EDTA, 50% formamide, 5× Denhardt’s solution, 4% dextran sulfate, 0.5% SDS, and 20 mg/ml heat-denatured salmon sperm DNA. The filters were washed twice with 2× SSC/0.1% SDS, once with 1× SSC/0.1% SDS, and twice with 0.1× SSC/0.1% SDS at 42°C.

Decay accelerating factor expression and miniPLAP in vitro translation assay of the development of ES cells. Trypsinized ES cells were cultured in bacterial dishes in medium without leukemia inhibitory factor (LIF), feeding every other day. Cells (2.5×10⁵) were harvested at 3, 6, and 9 days. Cells were stained for surface decay-accelerating factor (DAF) expression using rat anti-murine DAF monoclonal antibody (MAb) 2C6 [generously provided by Dr. Paul Morgan (Cardiff)] and FITC-labeled goat anti-rat immunoglobulin (Pharmingen). Stained cells were analyzed in a FACScan (Becton Dickinson) flow cytometer (5). Cells were stained without and with prior incubation with recombinant Bacillus thuringensis phosphatidyl inositol-specific phospholipase C (PI-PLC) (5). Rough microsomes and the miniPLAP in vitro translation assay were performed as described before (4).

In situ hybridization analysis. The in vivo expression of mGPAA1 mRNA was examined by in situ hybridization (ISH) as described previously (27, 29). For this purpose a 1,014-bp SmaI fragment of mGPAA1 cDNA was subcloned into the SmaI site of pBluescript II SK(−). Plasmids with the insert in both directions were prepared, and sense and antisense RNAs were generated by T7 RNA polymerase. After death, whole fetal mice were immediately fixed with 4% paraformaldehyde in 100 mM phosphate buffer at 4°C overnight. Consecutive sections of fetal mice were used for analysis. Tissue sections on silane-coated glass slides were fixed for 15 min at room temperature with 4% paraformaldehyde in 100 mM phosphate buffer and treated for 30 min at 37°C with 10 mg/ml protease K [in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. The sections were then incubated for 10 min at room temperature in 4% formaldehyde in 100 mM phosphate buffer, 0.2 M HCl, and 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). After these treatments, the sections were washed with phosphate buffer (pH 7.4) and gradually dehydrated in ethanol. Hybridization was carried out for 20 h at 50°C using 10 ng/ml tRNA, 0.05% heparin, 0.1% BSA, and 1% SDS. Subsequently, the sections were washed, and the ISH signals were detected by staining for overnight at room temperature with the alkaline phosphatase substrate solution of nitro blue tetrazolium chloride (Boehringer Mannheim). Each slide was examined by three experienced pathologists.

Isolation and analysis of 3T3 cell lines expressing antisense mGPAA1. We subcloned the 5′-terminal 550-bp fragment of mGPAA1 cDNA into pMAM2-BSD vector (Kaken Seiyaku, Japan) in an inverted direction: this fragment contains a dexamethazone-inducible promoter and a blasticidin-resistant gene (15). We transfected 3T3 cells with this construct and selected them with 8 μg/ml of blasticidin. Total RNA was extracted from isolated clones cultured in the medium containing 1 μg/ml of dexamethasone. Expression of 550-base antisense RNA was detected by Northern blot analysis with 32P-labeled mGPAA1 cDNA. Parental 3T3 cell lines and selected clones were stained with anti-Ly6 or CD24 monoclonal antibody/FITC-conjugated anti-mouse immunoglobulin. They were analyzed on the FACScan flow cytometer.
RESULTS

Isolation of murine GPAA1 cDNA. We obtained 36 positive plasmids from the EB library using the signal sequence method. The deduced amino acid sequence of one clone, 82D3, showed no homologies to any known sequences except yeast Gaa1 protein (yGaa1p) (10). When we screened a murine heart cDNA library with the 82D3 cDNA fragment, we obtained mGPAA1 cDNA and determined its nucleotide sequence (Fig. 1). mGPAA1 cDNA was ~2 kb in length, a size almost the same as that of the mRNA transcript detected in Northern blot analysis. The sequence around the predicted translation initiation site (CGCCATGGG) was consistent with the Kozak (16) consensus sequence. The overall sequence showed a long open reading frame encoding 621 amino acids. In the 3′-terminal untranslated region, there was a polyadenylation signal (AATAAA) 26 bp upstream of the poly(A) tract. Computer analysis showed that the deduced protein had a 47-amino acid signal sequence at its NH2 terminus (26), one cAMP- and cGMP-dependent protein kinase phosphorylation site, seven protein kinase C (PKC) phosphorylation sites, two putative N-glycosylation sites, and eight putative transmembrane domains. The overall deduced amino acid sequence was 91% identical to that of hGpaa1p (human Gpaa1 protein) (10) and 25% identical and 57% homologous to that of yGaa1p (8).

Genomic structure of mGPAA1 gene. We recovered only a single positive clone from a murine genomic library. This clone contained about 16 kb DNA, and it was confirmed by Southern blot analysis using 32P-labeled mGPAA1 cDNA. Restriction enzyme mapping showed that this clone had a 3.6-kb BamHI fragment. This BamHI fragment was the same length as a single positive band obtained by BamHI digestion of murine genomic DNA Southern blot analysis (data not shown). To confirm that the BamHI I fragment contains mGPAA1 genomic DNA and to determine the exact exon and intron locations, we determined nucleotide sequence of an 8.9-kb fragment encompassing the BamHI fragment. The analysis revealed that the mGPAA1 gene has 12 small exons and 11 small introns and that the BamHI I fragment contains all of the exons and introns (Fig. 2). A putative translation initiation site is present in the exon 1 and a termination site in exon 12. All exon-intron boundary sequences except the intron 8 sequence are in accordance with the GT-AG rule (Table 1).

Expression of mGPAA1 mRNA. At first we performed Northern blot analyses of murine tissues in fetal, neonatal, and adult stages to examine the expression of mGPAA1 mRNA. Strong expression of mGPAA1 was detected in murine fetal (Fig. 3A) and neonatal (Fig. 3B) tissues. mGPAA1 mRNA was detected as a single band, and its size was about 2 kb. The size was almost the same as that of the mGPAA1 cDNA that we isolated. In the adult, mGPAA1 also was expressed ubiquitously, but its expression levels differed among tissues. In general, they were weaker than in fetal and neonatal tissues.

To examine the expression of mGPAA1 mRNA in different cell types, we performed in ISH analysis of murine embryos. mGPAA1 was expressed ubiquitously, and we found mGPAA1 mRNA expression at high levels in the choroida of the fourth ventricle (Fig. 4, A–C), skeletal muscle cells (Fig. 4, D–F), osteoblasts of ribs (Fig. 4, G–I), and occipital bone (Fig. 4, J–L).

DAF expression and miniPLAP in vitro translation assay of the development of ES cells. We previously had shown that mGPAA1 mRNA expression decreased with the development of ES cells (10). We compared surface expression levels of the DAF that is a widely expressed GPI-anchored protein. We confirmed that DAF on the cells was GPI-anchored by showing that it could be released by PI-PLC and that levels of the
Fig. 4. In situ hybridizations of mGPAA1. Strong signals were detected in choroidae of the fourth ventricle (A–C), skeletal muscle (D–F), rib (G–I), and occipital bone (J–L). Hematoxylin-eosin staining sections are shown (left). Antisense and sense sections are also shown (middle and right, respectively). Arrows indicate strong signal of mGPAA1.

Fig. 5. Decay-accelerating factor (DAF) expression on embryonic stem (ES) cells. After removal of leukemia inhibitory factor, ES cells were harvested at 3, 6, and 9 days, at which time beating was observed. Histograms showing surface DAF levels on the cells are shown (A) and mean DAF fluorescence levels are given diagrammatically (B). The open lines (A) show the staining with nonrelevant isotype-matched control monoclonal antibody. Surface DAF levels progressively declined with the appearance of beating.
protein declined during the differentiation (Fig. 5, A and B). DAF protein decreased with mGPAA1 mRNA levels. We used the miniPLAP in vitro translation assay to examine the transamidation reaction directly. In this assay, COOH-terminal processing of 27.0-kDa prominiPLAP yields 24.7-kDa GPI-anchored mature miniPLAP. We found that in accordance with Northern blot data of mGPAA1 mRNA levels, the efficiency of the transamidation reaction went down with the appearance of beating ES cells (Fig. 6, A and B).

Analysis of function of cell lines expressing antisense mGPAA1. We prepared 3T3 cell lines expressing antisense mGPAA1 controlled by a dexamethasone-inducible promoter to clarify mGPAA1 function. We selected 12 clones with blasticidin, and we detected that 6 of them overexpressed 550-bp antisense mGPAA1 by Northern blot analysis. Ly6 and CD24 are known to be GPI-anchored proteins and to exist in 3T3 cells. We could detect both Ly6 and CD24 with monoclonal antibodies on the surface of parental 3T3 cells (Fig. 7A). Neither Ly6 nor CD24 could be detected on any of the six cell lines expressing antisense mGPAA1 (Fig. 7B). In conjunction with the above miniPLAP data, these results indicated that mGpaa1p participates in GPI-anchoring acting at the GPI transfer step like yGaa1p.

DISCUSSION

Several results suggest that the final step in GPI-anchored protein synthesis is accomplished by a transamidase (1, 20, 21); a transamidation mechanism for GPI attachment to protein has recently been confirmed in trypanosomes (28), but the mammalian cellular machinery of this GPI transamidase reaction is not yet defined. It is difficult to directly measure the activity of the transamidase in crude extracts because the transamidase requires two substrates, a proprotein and the GPI anchor donor (34). Recently, two components of the GPI transfer machinery have been identified based on the analysis of the two yeast mutants gaa1 (8) and gpi8 (2), both of which synthesize the complete GPI anchor precursor but fail to attach it to proteins. Overexpression of yGAA1 restored the ability of gaa1 mutant cells to anchor GPls to proproteins (8).

In this study, we have isolated mGPAA1 cDNA and genomic DNA by the signal sequence trap method. mGpaa1p has an NH2-terminal signal sequence like that of yGaa1p that consists of 47 amino acids and functions as an NH2-terminal signal sequence in this method. The nucleotide sequences of mGPAA1 and human GPAA1 showed no significant homology with any known genes, but the deduced amino acid sequences showed 25% identity and 57% homology with that of yGaa1p. mGPAA1 cDNA has a long open reading frame with short 5' and 3'-untranslated regions. A putative translation initiation site is fully consistent with Kozak (16) consensus, and a polyadenylation signal and a poly(A) tract exist at the 3' terminus. The size of the cDNA is almost the same as that of the mRNA detected on Northern blot analysis (Fig. 3). We
also determined the murine gene structure and compared its sequence with the cDNA sequence. Our data indicated that we isolated a full-length mGPAA1 cDNA.

There are several consensus motifs in the deduced proteins of mGPAA1 and hGPAA1. All of them were conserved in both Gpaa1p proteins. They have two putative N-glycosylation sites and seven PKC phosphorylation sites, but Ygaa1p has only three PKC phosphorylation sites. Unlike Ygaa1p, they have one cAMP- and cGMP-dependent protein kinase phosphorylation site. These putative phosphorylation sites may be important for the regulation of Gpaa1p function, and in higher eukaryotes, a cAMP- and cGMP-dependent protein kinase phosphorylation site might play an important role. Leucin zipper motif is also conserved, and this motif is known to take part in protein interaction. It may be important for the formation of transamidase complexes.

Southern blot analyses of murine genomic DNA suggested that the mGPAA1 gene is a single copy gene in the haploid murine genome. It also exists as a single gene in yeast (8). The mGPAA1 gene has 12 small exons and 11 small introns that are contained in a 3.6-kb Bam HI fragment. The mGPAA1 gene is small compared with other PIG genes that participate in GPI anchor synthesis.

mGPAA1 mRNA is expressed ubiquitously in the fetus, neonate, and adult (Fig. 3, A–C). mGPAA1 resembles housekeeping genes, and yGAA1 is necessary for yeast survival. In mammalian cells, GPI anchor-deficient cell lines survive, but PIG-A-deficient mice cannot be produced from PIG-A-deficient ES cells (6, 14). These results suggest that GPIs or GPI-anchored proteins are necessary to form particular organs or tissues in the early embryonic stage. In our experiments, mGPAA1 mRNA expression levels were higher at the embryonic stage than at the adult stage, and in the in vitro differentiation system of ES cells, mGPAA1 mRNA levels decreased in the course of development (10). Moreover, GPI-anchored DAF expression and the transamidation efficiency directly measured by miniPLAP assay decrease with the differentiation of ES cells (Figs. 5 and 6). These results also support the above proposition.

ISH analysis elucidated the high expression of mGPAA1 mRNA at the cellular level. It revealed that mGPAA1 mRNA is expressed abundantly in choroida of the fourth ventricle of brain. The reason for the high expression in muscle cells and osteoblasts is unknown.

Recently, another protein, Gpi8p, which is essential for GPI transfer, has been characterized, and the yeast gpi8 mutant cannot be rescued by overexpression of yGAA1 (2). Expression of yGPI8 rescues the yeast gpi8 mutant, and expression of hGPI8 rescues class K cells (37). These results indicate that at least two gene products participate in the transamidation reaction both in mammalian cells and in yeast. Gpi8p has homology to a family of plant endopeptidases, one of which has transamidase activity (37). Gpi8p may be a catalytic component of transamidase machinery complex, and Gpaa1p possibly could function to hold COOH-terminal signal sequences of nascent proteins.

Previously, we showed that transient overexpression of antisense hGPAA1 significantly reduced the production of a reporter GPI-anchored protein in human K562 cells. So, we selected 3T3 cell lines expressing antisense mGPAA1 under the dexamethasone-inducible promoter. GPI-anchored Ly6 and CD24 expressions on the cell surface were completely inhibited by antisense mGPAA1 (Fig. 7). These results suggested that mGpaa1p has a function in the GPI anchor attachment step similar to yeast Gaa1p.

In summary, we have isolated mGPAA1 cDNA and the mGPAA1 gene. Gpaa1p protein presumably plays an important role with Gpi8p in the transfer step of GPI anchor attachment to protein. Their precise functions need further investigation.

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The sequences reported in this paper have been deposited in the European Molecular Biology Laboratory, GenBank, and DNA Data Bank of Japan nucleotide sequence databases with the following accession numbers: AB006970 and AB006971.

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