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Molecular cloning of human homolog of yeast *GAA1* which is required for attachment of glycosylphosphatidylinositols to proteins

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Abstract Anchoring proteins to cell surface membranes by glycosylphosphatidylinositols (GPIs) is important. We have isolated a component of the putative transamidase machinery, hGaa1p (human GPI anchor attachment protein). *hGAA1* cDNA is approximately 2 kb in length and codes 621 amino acids. The amino acid sequence of hGaa1p is 25% identical and 57% homologous to that of yeast Gaa1p. Moreover, Kite-Dolittle hydrophobicity plots of both proteins show marked similarity. *hGAA1* gene is expressed ubiquitously and mRNA levels are higher in the undifferentiated state. Overexpression of antisense hGAA1 in human K562 cells significantly reduced the production of a reporter GPI-anchored protein.

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Key words: Gaa1; GPI anchor; Transamidase; Glycosylphosphatidylinositol; Antisense; Signal sequence trap

1. Introduction

Posttranslational glycosylphosphatidylinositol (GPI) anchor attachment serves as a general mechanism for linking proteins to the cell surface membrane [1–3]. The mechanism of mammalian GPI biosynthesis has been studied using mutant cells that are defective in different steps of the biosynthetic pathway. Important among these mutants have been GPI-defective murine T-cell lymphomas selected by their inability to express Thy-1 on their surfaces [4]. These mutants, grouped into different complementation classes, A, B, C, E, F, and H, transcribe the Thy-1 gene normally but retain Thy-1 protein intracellularly. Classes A, C, and H are all defective in the first step, synthesis of *N*-acetylglucosaminyl phosphatidylinositol [5,6]. Class E is not able to synthesize dolichol-phosphoryl-mannose, the mannose donor for GPI synthesis [6,7]. Class B cannot incorporate distal α1-2-linked mannose into the glycan core [6] and class F is unable to add terminal ethanolamine phosphate to this third mannose residue [5,6,8]. Additional complementary mutant lines representing other steps have been *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) treated human K562 erythroleukemic cell mutants, classes J and K [9,10]. Class J is defective at the second step, deacetylation of *N*-acetyl-D-glucosamine-inositol phospholipid to glucosamine-inositol phospholipid [10]. Recently, two other mutants of Chinese hamster ovary cells having defects in the same second step were reported [11,12]. One was designated class L. Class L may be identical to class J. Many of the genes responsible for these defects have been isolated including *PIG* (phosphatidyl inositol glycan)-*A* [13], *PIG-B* [14], *PIG-C* [15], *PIG-F* [16], *PIG-H* [17], and *PIG-L* [12]. In contrast to the above mutants all of which are defective in GPI assembly, in the class K mutant, the complete GPI precursor is formed, but a defect in the substitution of GPIs for COOH-terminal signal sequences is responsible for its inability to display GPI-anchored proteins [10,18].

As in mammalian cells, a number of mutants in yeast which cannot express GPI-anchored proteins have been described and several of the affected genes identified [19–21]. *SPT14*/*GPI3* and *GPI2* are homologs of mammalian *PIG-A* [22] and *PIG-C* [15], respectively. The yeast *GAA1* (*yGAA1*) gene has been isolated and characterized using a yeast GPI-anchored protein deficient mutant termed *gaa1* [23]. *gaa1* can synthesize complete GPI precursors, but it cannot express GPI-anchored proteins on the cell surface [23]. Another yeast gene *yGPI8* has been isolated from a second yeast mutant termed *gpi8* with a phenotype identical to that of *gaa1* [24]. Thus *gaa1* and *gpi8* mutant cells have defects similar to mammalian class K mutant cells. Recent studies have shown that mammalian class K mutant cells have a defect in the activity of the transamidase [10,18]. The gene responsible for this defect, i.e. the *PIG-K* gene, recently has been shown to correspond to *hGPI8* [25].

2. Materials and methods

2.1. The signal sequence trap method

Embryonic stem (ES) cells were A3.1 derived from the 129/SvJ mouse strain [26]. poly $(A)^+$ RNA was extracted from embryoid bodies (EB) that had been differentiated for 6 days. The signal sequence method was performed as described before [27]. In brief, 5′ termini-enriched EB cDNAs were ligated to the pcDL-SRα-Tac (3′) vector in the same orientation with the Tac (α chain of the human interleukin-2 receptor) cDNA to obtain an expression library. The COS cells that were transfected by new secretory signal sequence fusioned Tac plasmids expressed Tac fusion proteins on the cell surface, and they were detected with anti-Tac antibody and FITC-conjugated second antibody. DNA sequences were determined on both strands by the 373A DNA sequencer using the fluorescent dideoxy terminator sequencing kit (Perkin Elmer). DNA sequences and deduced protein sequences were compared with sequences in the GenBank database for homology.

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Abbreviations: GPI, glycosylphosphatidylinositol; Gaa, GPI anchor attachment; PIG, phosphatidyl inositol glycan

The sequence reported in this paper has been doposited in the GenBank, EMBL and GenBank nucleotide sequence databases with the accession number AB006969.

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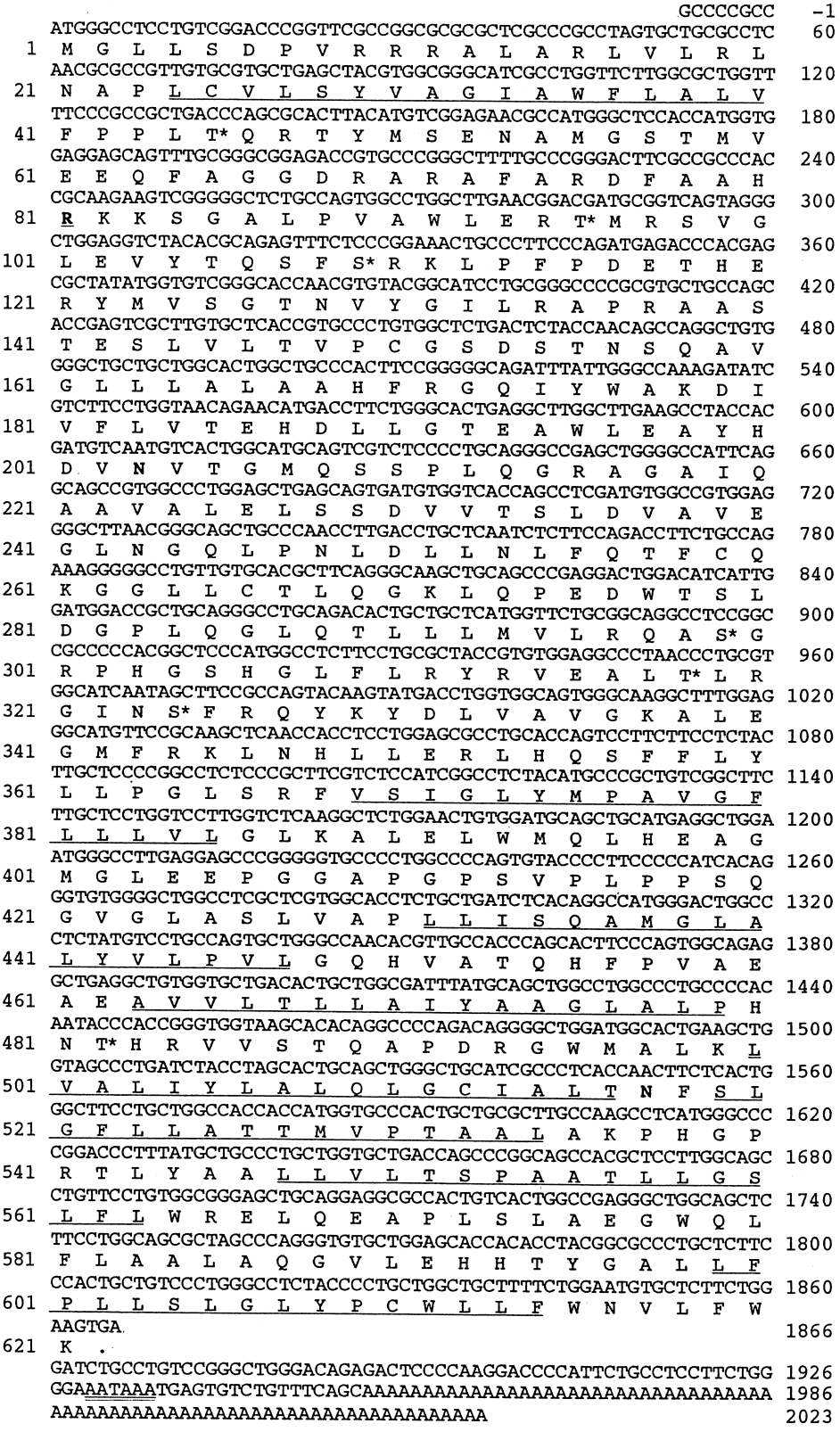

Fig. 1. DNA and amino acid sequence of *hGAA1*. The nucleotide sequence of *hGAA1* cDNA is shown with nucleotide positions indicated in the right. The deduced amino acid sequence of *hGAA1* is shown in single-letter code, with the amino acid numbers in the left. The putative eight transmembrane domains are underlined. Two N-linked glycosylation sites are bold. A phosphorylation site by cAMP- and cGMP-dependent protein kinase is bold and underlined. Protein kinase C phosphorylation sites are indicated with asterisks. Four leucins in leucin zipper pattern are indicated by italic. A polyadenylation signal is doubly underlined. The GenBank, EMBL and DDBJ accession number is AB006969.

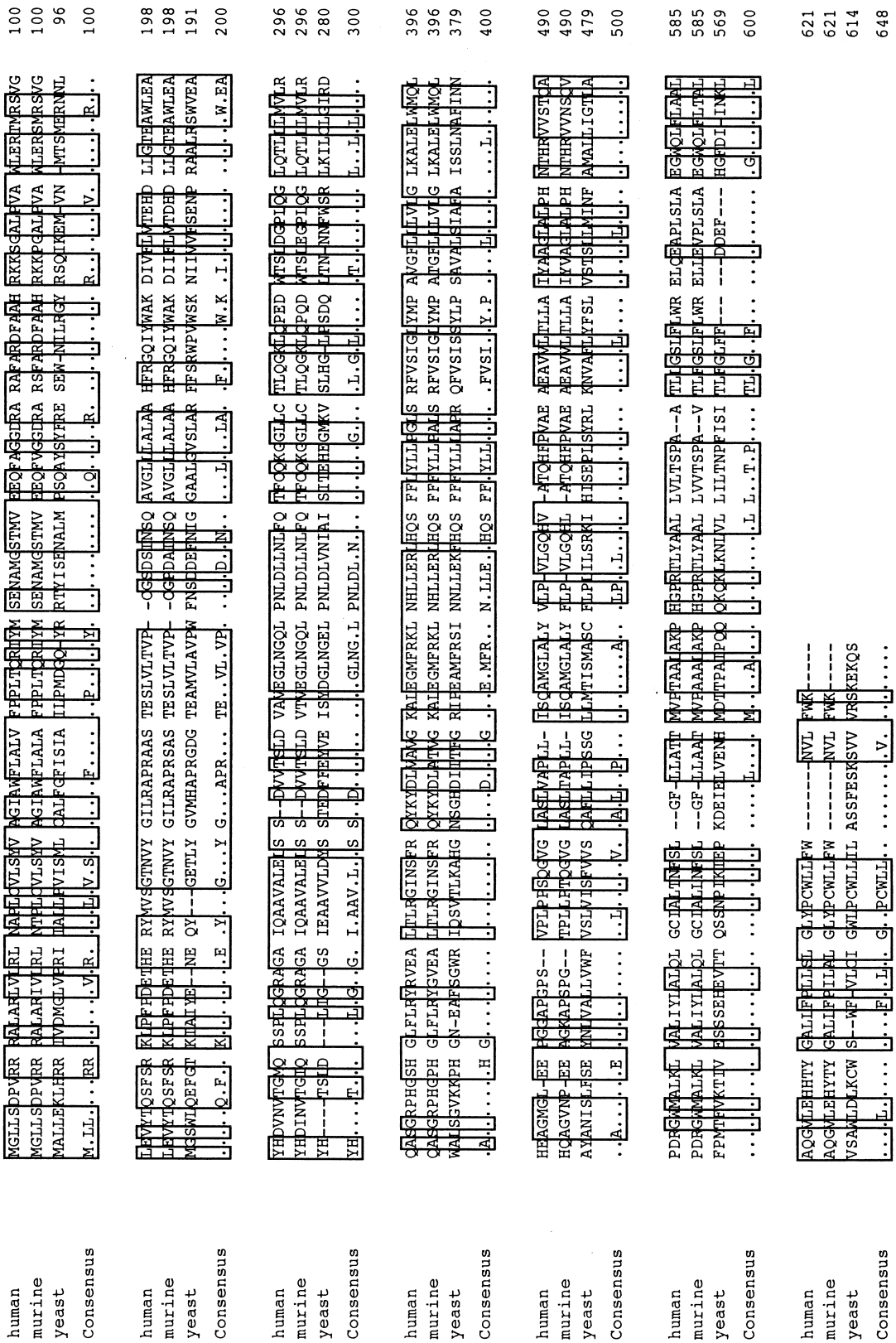

Fig. 2. Comparison of hGaa1p and yGaa1p. A: Sequence comparison of human, murine and yeast Gaa1 protein. The amino acid numbers are written in the right. Identical or homologous amino acids are enclosed by open boxes. Identical amino acids are indicated at the fourth lane as consensus amino acids. B: Kyte-Dolittle hydrophobicity plots of hGaa1p and yGaa1p. Longitudial axis indicates hydrophobicity score. Hydrophobic and hydrophilic amino acids show positive and negative scores, respectively.

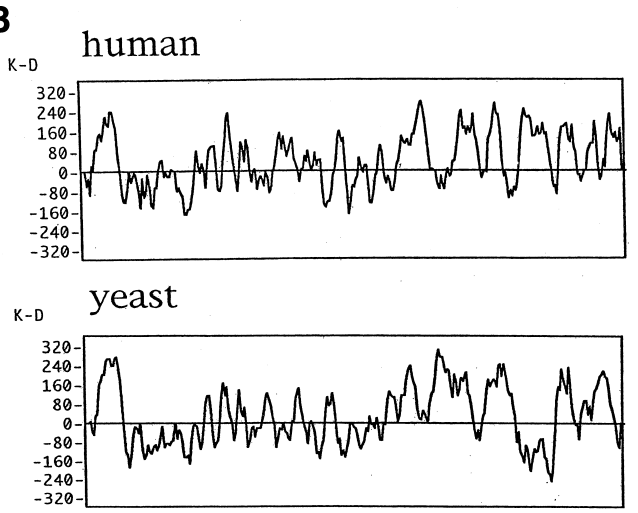

Fig. 2 (*continued*).

2.2. Murine and human cDNA library screening

Approximately 1.0×10^6 plaques of murine heart cDNA library in Uni-ZAP XR Vector (Stratagene) were screened. Plasmids were excised from phage vectors in vivo by the ExAssist helper phage. We next screened human fetal heart cDNA library in λgt11 (Clontech) using murine *GAA1* cDNA. Phage DNA was purified from isolated clones, digested with *Eco*RI, and subcloned into pBluescript II SK^+ (Stratagene).

2.3. Northern blot analysis

Expression of *hGAA1* mRNA in human tissues was examined using Human fetal MTN blot and Human adult MTN blot (Clontech). The filters were hybridized with ^{32}P-labeled human *GAA1* cDNA at 42°C for 12 h in 5×SSPE, 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. Total RNA was extracted from embryoid bodies, rat fetal and neonatal heart, and adult ventricle and atrium using RNAzolB (Biotecx Laboratories). Twenty micrograms of total RNA were separated on 1.0% formaldehyde-agarose gel and transferred to nylon membrane filters, Hybond-N. The filters were hybridized with ^{32}P-labeled murine *GAA1* cDNA.

2.4. Transient transfection of K562 cells

We subcloned a full length *hGAA1* cDNA into pCDNA3 vector (Invitrogen) in both directions. K562 cells (6×10^6 at 5–8×10^5/ml) were incubated overnight in RPMI 1640 containing 10% heat inactivated newborn calf serum, washed twice with serum-free RPMI 1640, and readjusted to 5×10^6/ml. One milliliter of washed cells was inoculated with 1 MOI/cell of VTF7.3 virus in RPMI 1640 containing 2% newborn calf serum, and the cells infected for 1 h by shaking at 15 min intervals at 37°C. Infected cells were washed with serum-free RPMI followed by HeBS transfection buffer, and after adjustment to 10^6 cells in 0.8 ml of HeBS, 30 μg of CD8-DAF/pCDNA1 and 10 mg salmon sperm DNA were added with or without 10 μg of the test plasmid. The mixture was incubated on ice for 10 min and the DNA introduced by electroporation at 250 V and 960 mF in a Gene-Pulser (Bio-Rad Laboratories). Electroporated cells were cultured in complete RPMI 1640. After 48 h they were stained with anti-CD8 mAB/FITC-conjugated anti-mouse Ig and propidium iodide and analyzed on a FACStar^{Plus} (Becton Dickson) flow cytometer.

3. Results

3.1. Isolation of signal sequence containing proteins from an EB library

By screening 7800 clones, we obtained 36 positive plasmids from the EB library by the signal sequence method. All positive clones were sequenced and their sequences were searched for in the GenBank database. The deduced amino acid sequence of one clone, 82D3, showed no homologies to any known sequences except yGaa1p.

3.2. Isolation and sequencing of human GAA1 cDNA

With the use of 82D3 cDNA we screened a murine heart cDNA library and obtained two full length murine *GAA1* cDNAs which we termed M1 and M2 (data not shown). Using M1 we next screened a human heart cDNA library and obtained three clones which we termed H1, H10, and H18. Nucleotide sequences of the three cDNAs were determined and the analysis revealed that H18 encompassed a 1.6 kb fragment of the 5′-terminal region and H1 an overlapping 1.5 kb fragment of the 3′-terminal region of an overall human cDNA corresponding to murine *GAA1* cDNA. H10 overlapped the central 1.0 kb region of H1. A composite cDNA designated H118 was prepared by digesting H1 and H18 with *Eco*RV and ligating them. It is approximately 2 kb in length (Fig. 1), a size almost the same as that of mRNA detected in Northern blot analysis. The sequence around the predicted translation initiation site (CGCC*ATG*GG) is consistent with the Kozak consensus sequence. The H118 cDNA sequence has a long open reading frame consisting of 621 amino acids. In the 3′-terminal untranslated region, there is a polyadenylation signal (AATAAA) 17 bp upstream of the poly (A) tract. Computer analysis showed that the deduced protein has a 47 amino acid secretory signal sequence at the NH_2 terminus, one cAMP- and cGMP-dependent protein kinase phosphorylation site, one leucine zipper pattern, two putative *N*-glycosylation sites, and eight putative transmembrane domains.

The overall deduced amino acid sequence shows significant homology with that of yGaa1p (Fig. 2A). The translated H118 cDNA sequence is 25% identical and 57% homologous to that of yGaa1p. Moreover, the Kite-Dolittle hydrophobicity plot of the deduced protein corresponds closely to that of yGaa1p (Fig. 2B). We thus named this human cDNA as *hGAA1* (human *GAA1*).

3.3. Expression of hGAA1

We examined expression of *hGAA1* mRNA in various tis-

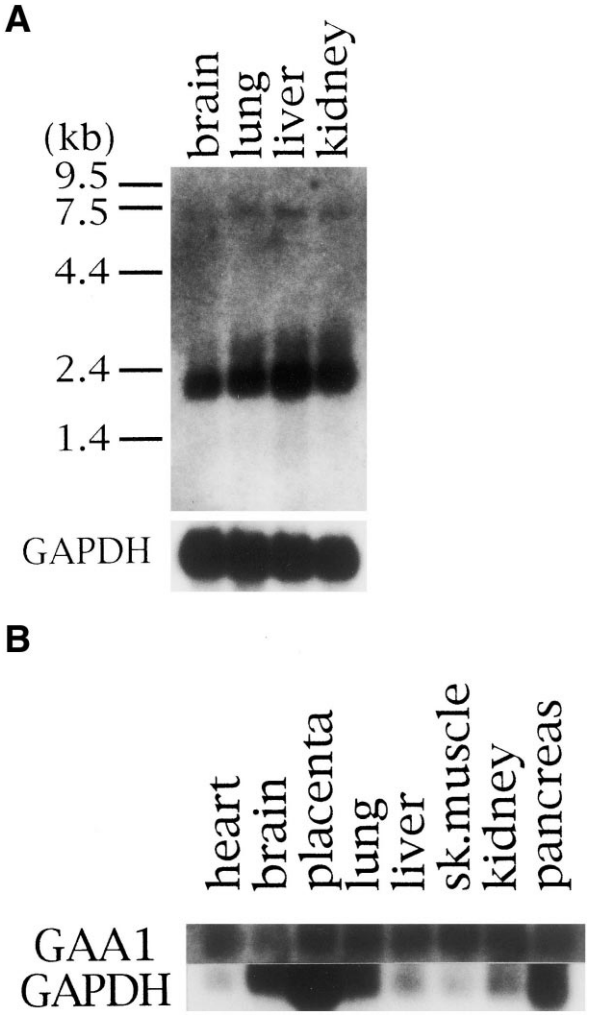

Fig. 3. Expression of *GAA1* mRNA in various tissues. Northern blot analysis of *hGAA1* in human fetal (A) and adult (B) tissues (human fetal and adult MTN blot). Two micrograms of poly $(A)^+$ RNA isolated from various human fetal and adult tissues were hybridized with ^{32}P-labeled *hGAA1* cDNA probe.

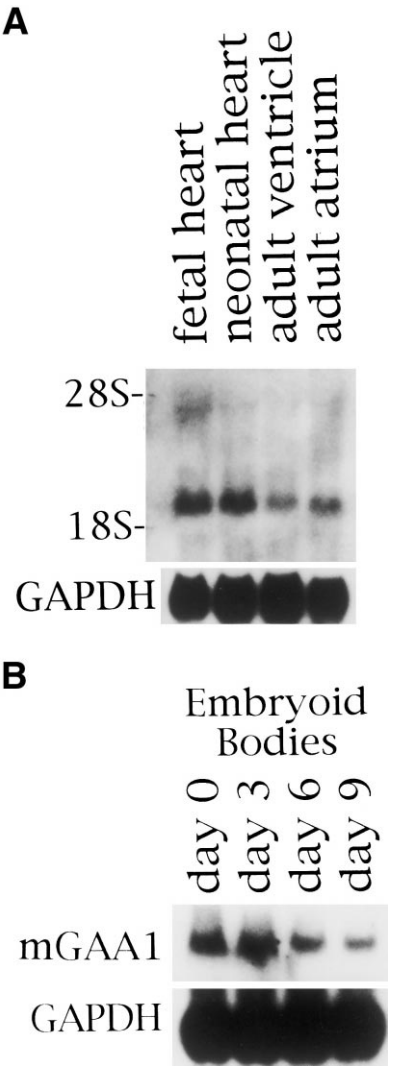

Fig. 4. Expression of *GAA1* mRNA in the developmental stage. A: Northern blot analysis of rat *GAA1* in rat fetal and neonatal heart and adult ventricle and atrium. B: Northern blot analysis of murine *GAA1* in undifferentiated embryonic stem cells and differentiated embryoid bodies. Twenty micrograms of total RNA were hybridized with ^{32}P-labeled murine cDNA probe.

sues of the human fetus and adult. A single band of ~2 kb in length was detected in all tissues of the human fetus (Fig. 3A) and adult (Fig. 3B) that were examined. Expression levels were generally higher in fetal than in adult tissues. We next examined alterations of expression levels of rodent *GAA1* mRNA during development. In rats, *GAA1* mRNA was abundantly expressed in the fetal and neonatal hearts as compared with the adult heart (Fig. 4A). When ES cells are cultured in suspension without LIF, they develop into EB and finally various cell types including cardiac myocytes and hematopoietic cells [28]. This in vitro differentiation system reflects the in vivo situation and is useful to examine the very early stage of the embryo [29]. In EB, mRNA levels were high in the undifferentiated state and gradually decreased with development (Fig. 4B).

Table 1
Percent GPI-anchored CD8 positive cells

	Experiment		
	1	2	3
Antisense	1.64 ± 0.07	3.40 ± 0.7	10.33 ± 0.2
Control	4.10 ± 0.35	6.50 ± 0.07	
Sense		7.30 ± 1.0	15.10 ± 2.7

In all experiments, antisense *hGAA1* cDNA significantly decreased the percent of GPI-anchored CD8 positive cells.

3.4. Transient transfection of K562 cells

To examine whether hGaa1p functions in GPI anchoring, we transfected antisense *hGAA1* cDNA into K562 cells with the GPI-anchored protein reporter cDNA, CD8-DAF (Table 1). At the first experiment, the percentage of GPI-anchored CD8 positive cells following transfection with CD8-DAF/pCDNA1 was 4.10 ± 0.35 (Fig. 5, upper two panels). Overexpression of antisense *hGAA1* cDNA strongly inhibited this expression to less than half (1.64 ± 0.07) of control levels (Fig. 5, lower two panels). A second experiment was performed using both antisense and sense *hGAA1* cDNA. Overexpression of sense *hGAA1* cDNA had a small but not significant effect on the expression of GPI-anchored CD8, but overexpression of antisense cDNA again significantly decreased it (control,

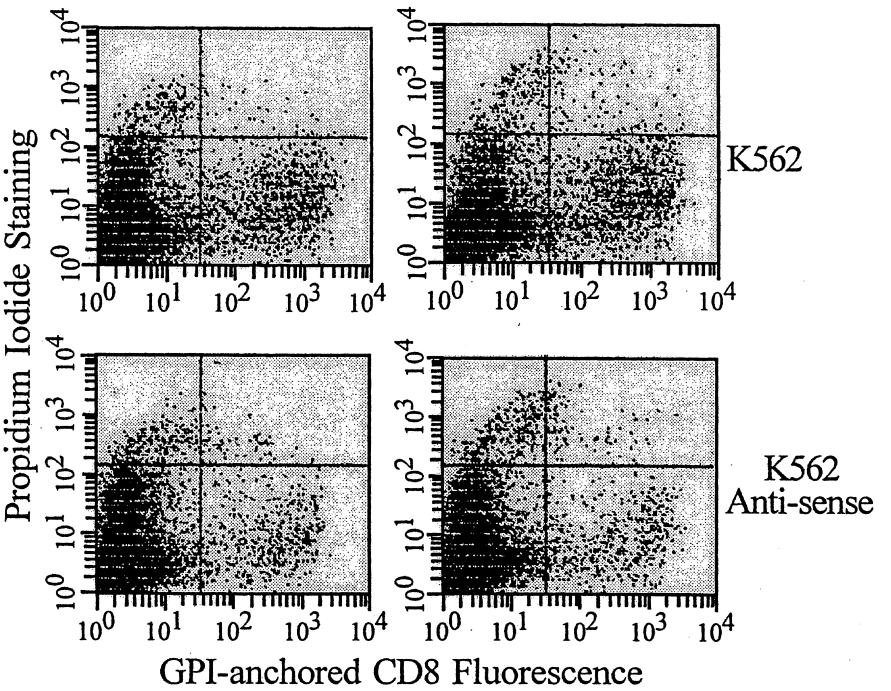

Fig. 5. Functional analysis of *hGAA1* by overexpressing antisense *hGAA1* cDNA. K562 cells transfected by the GPI-anchored reporter cDNA without (upper two panels) or with antisense *hGAA1* cDNA (lower two panels) were analyzed by FACS. Longitudial axis indicates the intensity of propidium iodide staining and horizontal axis indicates the intensity of GPI anchored CD8 fluorescence, which reflects the amount of GPI-anchored reporter protein. Overexpression of antisense *hGAA1* cDNA decreased the cell number of GPI-anchored CD8 fluorescence positive cells presented in the right of threshold.

6.50 ± 0.07; antisense, 3.40 ± 0.07). A third experiment using sense and antisense constructs reproduced the inhibitory effect of antisense *hGAA1* cDNA (sense, 15.10 ± 2.27; antisense, 10.33 ± 0.20). These results indicate that hGaa1p functions in GPI anchoring presumably acting at the GPI transfer step as has been shown for yGaa1p.

4. Discussion

Although several results suggest that the final step in GPI-anchored protein synthesis is accomplished by a transamidase [30–32], the cellular machinery involved in this GPI transamidase reaction is not yet defined. It is difficult to directly measure the activity of the transamidase in crude extracts [2]. Recently, two components of the GPI transfer machinery have been isolated from two yeast mutants *gaa1* [23] and *gpi8* [24] both of which synthesize the complete GPI anchor precursor, but do not attach it to proteins. Overexpression of the first of these proteins Gaa1p (the subject of this study) enabled *gaa1* mutant cells to GPI anchor proteins and improved their ability to attach anchors to nascent polypeptides of GPI-anchored proteins with mutant anchor attachment sites [23].

In this study, we isolated a human homolog of yGaa1p by the signal sequence trap method. This method is a unique cloning strategy which uses NH_2-terminal signal sequences. With this method, not only secretory molecules, but also ER or Golgi proteins containing NH_2-terminal hydrophobic sequences have been isolated [27]. In fact, we have isolated many such proteins including interferon α/β receptor and signal sequence peptidase α in this way. A computer analysis showed that hGaa1p has an NH_2-terminal signal sequence like that of yGaa1p.

The nucleotide sequence of *hGAA1* showed no significant homology with any known genes, but the deduced amino acid sequence showed 28% homology with that of yeast Gaa1p. *hGAA1* cDNA has a long open reading frame with short 5′ and 3′ untranslated regions. The putative translation initiation site is fully consistent with the Kozak consensus and there is a polyadenylation signal and a poly (A) tract at the 3′ terminus. The size of the cDNA is almost the same as that of the mRNA detected in Northern blot analysis (Fig. 3A). In addition, the NH_2-terminal sequences between hGaa1p and yGaa1p are also highly conserved. We thus believe that we have isolated cDNA sequence corresponding to full length *hGAA1* mRNA.

There are several consensus motifs in the deduced protein of *hGAA1*. All of them are conserved in both human and murine Gaa1p. The second phosphorylation site by protein kinase C is homologous, threonine in human and serine in murine, and other motifs are identical. Kite-Dolittle hydrophobicity analysis revealed that hGaa1p has eight putative transmembrane domains, while yGaa1p has only six transmembrane domains. This difference could be due to the program algorithm, because some putative transmembrane domains were assigned at different places by different analyses. Both hGaa1p and yGaa1p have two putative *N*-glycosylation sites. hGaa1p has seven protein kinase C phosphorylation sites and yGaa1p has three sites. Only hGaa1p has a cAMP- and cGMP-dependent protein kinase phosphorylation site. These phosphorylation sites could be important for regulation of hGaa1p function.

hGAA1 mRNA is expressed ubiquitously in human fetus and adult. The yeast *gaa1* mutant cannot survive in normal conditions [23] as GPI anchoring is indispensable for yeast but apparently not for mammalian cells. In the course of development, *GAA1* mRNA expression levels are higher in the em-

bryonic stage than in the adult. In the rodent heart, mRNA is abundant in the fetus and neonate, and it is low in the adult. Moreover, in the in vitro differentiation system of ES cells, *GAA1* mRNA levels decreased along with development. Under differentiation conditions, ES cells differentiate into many cell types and lose their proliferative ability. These results suggest that GPI anchoring is important in the early differentiating stage.

We examined the function of hGaa1p by overexpressing antisense *hGAA1* cDNA in human K562 cells. Although overexpression of sense *hGAA1* only slightly increased the production of our GPI-anchored reporter protein, CD8-DAF, overexpression of antisense *hGAA1* cDNA significantly decreased its production (Fig. 5, Table 1). These results suggest that hGaa1p has functional activity that would be consistent with that of yGaa1p. A *hGPI8* mutant would be needed to formally validate this proposal.

In summary, we have isolated a human homologue of *yGAA1* cDNA. Its protein product presumably is involved in the transfer step of GPI anchor attachment to protein. Its precise mechanism of action remains to be determined.

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