Molecular Biology of the Cell
Vol. 11, 1523–1533, May 2000

Gaa1p and Gpi8p Are Components of a Glycosylphosphatidylinositol (GPI) Transamidase That Mediates Attachment of GPI to Proteins

Kazuhito Ohishi,* Norimitsu Inoue,* Yusuke Maeda,* Junji Takeda,* Howard Riezman,† and Taroh Kinoshita*‡

*Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; and †Biozentrum, University of Basel, Basel CH-4056, Switzerland

Submitted December 16, 1999; Accepted February 17, 2000
Monitoring Editor: Guido Guidotti

Many eukaryotic cell surface proteins are anchored to the membrane via glycosylphosphatidylinositol (GPI). The GPI is attached to proteins that have a GPI attachment signal peptide at the carboxyl terminus. The GPI attachment signal peptide is replaced by a preassembled GPI in the endoplasmic reticulum by a transamidation reaction through the formation of a carbonyl intermediate. GPI transamidase is a key enzyme of this posttranslational modification. Here we report that Gaa1p and Gpi8p are components of a GPI transamidase. To determine a role of Gaa1p we disrupted a *GAA1/GPAA1* gene in mouse F9 cells by homologous recombination. *GAA1* knockout cells were defective in the formation of carbonyl intermediates between precursor proteins and transamidase as determined by an in vitro GPI-anchoring assay. We also show that cysteine and histidine residues of Gpi8p, which are conserved in members of a cysteine protease family, are essential for generation of a carbonyl intermediate. This result suggests that Gpi8p is a catalytic component that cleaves the GPI attachment signal peptide. Moreover, Gaa1p and Gpi8p are associated with each other. Therefore, Gaa1p and Gpi8p constitute a GPI transamidase and cooperate in generating a carbonyl intermediate, a prerequisite for GPI attachment.

INTRODUCTION

Numerous eukaryotic cell surface proteins are anchored to the membrane via a covalently attached glycosylphosphatidylinositol (GPI). Posttranslational attachment of GPI is essential for expression of those proteins on the cell surface. This type of membrane anchoring is widely used in all eukaryotic organisms.

In mammalian cells, more than 100 cell surface proteins with various sizes and functions are GPI-anchored (Low, 1989; Kinoshita *et al.*, 1995). At the cellular level, GPI-anchoring is not essential, and many GPI-deficient mutant cell lines have been established (Takeda and Kinoshita, 1995), indicating roles of GPI-anchored proteins in cell-to-cell interactions rather than cell growth itself. At the levels of tissues and the whole body, GPI-anchoring is critical. Keratinocyte-specific disruption of one of the GPI biosynthesis genes, *PIG-A*, demonstrated that GPI is essential for normal development of skin (Tarutani *et al.*, 1997). Disruption of *PIG-A* gene in the whole body resulted in embryonic lethality (Kawagoe *et al.*, 1996; Nozaki *et al.*, 1999). A human disease paroxysmal nocturnal hemoglobinuria is caused by somatic mutation of the *PIG-A* gene occurring in the multipotential hematopoietic stem cell (Takeda *et al.*, 1993).

In *Saccharomyces cerevisiae*, GPI is essential for growth (Leidich *et al.*, 1995). Analysis of the *S. cerevisiae* genome demonstrated that of ~6200 ORFs, ~60 encode GPI-anchored proteins (Caro *et al.*, 1997). Many of these are cell wall proteins. They are first synthesized and transported to the plasma membrane in the GPI-anchored form and then are incorporated into cell wall glucan after cleavage of the GPI portion (Lu *et al.*, 1995; Kollar *et al.*, 1997).

GPI-anchored proteins are formed in the endoplasmic reticulum (ER) from a preformed GPI and a protein precursor (Kinoshita *et al.*, 1995; Udenfriend and Kodukula, 1995). Proteins that are to be GPI-anchored have two signal peptides (Udenfriend and Kodukula, 1995). One is an amino-terminal signal peptide that directs translocation across the ER membrane. The other is a C-terminal signal peptide that directs attachment of the GPI anchor. Shortly after translation, the C-terminal GPI attachment signal peptide is recognized by a GPI transamidase that cleaves the signal and replaces it with GPI.

The amino acid to which GPI is attached is termed the ω site (Gerber *et al.*, 1992), and it must have a small side chain

‡ Corresponding author. E-mail address: tkinoshi@biken.osaka-u.ac.jp.

(Micanovic *et al.*, 1990; Moran *et al.*, 1991; Nuoffer *et al.*, 1993). The second residue carboxyl terminal to the ω site (ω+2) must also be a small amino acid, whereas the ω+1 site can be any amino acid except proline and tryptophan (Gerber *et al.*, 1992). The ω+2 site is followed by a stretch of hydrophilic amino acids, usually 5–7 residues, and a hydrophobic segment of 12–20 amino acids (Furukawa *et al.*, 1997). These are characteristics of the GPI attachment signal peptide, but there is no consensus sequence. The GPI transamidase is proposed to bind to the GPI attachment signal peptide and attack the carbonyl group of ω site amino acid with its catalytic site to release the signal peptide and generate a carbonyl intermediate between a precursor protein and the enzyme. GPI is then presented to this intermediate, whose amino group in the terminal ethanolamine would attack the intermediate to complete the transamidation reaction (Udenfriend and Kodukula, 1995; Sharma *et al.*, 1999).

The GPI transamidase that mediates GPI attachment has not been clearly characterized. Two *S. cerevisiae* mutants, *gaa1* (Hamburger *et al.*, 1995) and *gpi8* (Benghezal *et al.*, 1996), are defective in the attachment of GPI to proteins. *GPI8* encodes a protein with homology to members of a family of cysteine proteases (Benghezal *et al.*, 1996), one of which, a jack bean asparaginyl endopeptidase, showed transamidase activity in vitro (Abe *et al.*, 1993). A human mutant cell line termed class K that is defective in attachment of GPI (Mohney *et al.*, 1994; Chen *et al.*, 1996) is due to a defect in the human *GPI8* gene (Yu *et al.*, 1997). Microsomal membranes of class K cells did not have GPI transamidase activity (Chen *et al.*, 1996; Yu *et al.*, 1997). It was therefore suggested that Gpi8p is a component of the GPI transamidase (Benghezal *et al.*, 1996; Yu *et al.*, 1997). On the other hand, Gaa1p has no homology to other proteins in the databases, so it is not possible to predict its function. In the present investigation, we demonstrate that Gaa1p and Gpi8p form a protein complex, that Gaa1p is required for a precursor protein to form a carbonyl intermediate with the GPI transamidase, and that a conserved cysteine residue of Gpi8p is involved in cleavage of the signal peptide.

MATERIALS AND METHODS

Cells

Mouse F9 and human K562 cells were obtained from the American Type Culture Collection. The class K mutant was a gift from Dr. M. E. Medof (Case Western Reserve University). Mouse EL4 and its class F GPI-anchor–deficient mutant line Thy-1$^-$f were provided by Dr. R. Hyman (Salk Institute). They were cultured in high glucose DMEM supplemented with 10% FCS. Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium containing 10% FCS.

Expression Plasmids

All expression plasmids were constructed on pMEPyori in which the expression of the cloned insert is driven by SRα promoter (Ohishi *et al.*, 1996). We cloned the human *GAA1* cDNA into pMEPyori (pMEPyori–hGAA1) (Inoue *et al.*, 1999). A full-length human *GPI8* cDNA was obtained by ligation of the following two fragments at a unique *Msc*I site: an EST I.M.A.G.E. Consortium clone 33372 containing most of the ORF and a 5′ RACE product containing the missing region, which was amplified from placental mRNA. The resulting full-length human *GPI8* cDNA was cloned into pMEPyori (pMEPyori–hGPI8). The yeast *GPI8* ORF was amplified by PCR and cloned into pMEPyori (pMEPyori–yGPI8). Amino-terminally FLAG-tagged human *GAA1* and carboxyl-terminally GST-tagged human and yeast *GPI8*s were constructed as follows. To obtain pMEPyori-FLAG–hGAA1, a PIG-A–coding fragment of pMEPyori FLAG–PIG-A (Maeda *et al.*, 1998) was replaced with a PCR-amplified *Sal*I–*Eco*RV fragment containing the 5′ portion of human *GAA1* in which an initiation codon was substituted for a *Sal*I site and an *Eco*RV–*Not*I fragment containing the 3′ portion of human *GAA1* excised from pMEPyori–hGAA1. pMEPyori-HA–hGAA1 was obtained in a similar way. We obtained pMEPyori-hGPI8–GST by assembling three fragments on pMEPyori, an *Eco*RI–*Nhe*I fragment containing the 5′ portion of human *GPI8* from pMEPyori–hGPI8, a PCR-amplified *Nhe*I–*Mlu*I fragment containing the 3′ portion of human *GPI8* in which a *Mlu*I site was substituted for a stop codon, and a *Mlu*I–*Xba*I fragment from pMEEB-PIG-A–GST (Watanabe *et al.*, 1998) encoding GST. pMEPyori-yGPI8–GST was obtained in a similar way by assembling an *Eco*RI–*Bsp*119I fragment from pMEPyori–yGPI8, a PCR-amplified *Bsp*119I–*Mlu*I fragment and the GST fragment derived from pMEEB-PIG-A–GST. Amino-terminally FLAG-tagged human *GPI8* (pMEPyori-FLAG–hGPI8) was constructed by insertion of an HA epitope between Ile30 and Glu31 of human *GPI8* by means of oligonucleotide-directed mutagenesis. FLAG- and GST-tagged microsomal aldehyde dehydrogenase (msALDH) were described previously (Maeda *et al.*, 1998). Tagged versions of human Gaa1p and Gpi8p were functional because their cDNAs complemented *GAA1*-knockout cells and class K cells, respectively, to the same extent as cDNAs for nontagged counterparts. pMEpuro was constructed by cloning PGKpuro cassettes into the *Hin*dIII site of pME vector and used for the establishment of class K cells stably expressing GST-tagged Gpi8ps. Sequences of primers used are available on request.

Establishment of Mouse GAA1-Knockout F9 Cells

Targeting vectors were constructed as follows. A 7-kilobase (kb) *Bam*HI and blunt-ended *Xba*I fragment of mouse *GAA1* was cloned into *Bam*HI and blunt-ended *Eco*RI sites of pPNT (a gift from Dr. R. Mulligan, Harvard Medical School) (Tybulewicz *et al.*, 1991). A *Not*I–*Xho*I fragment containing a 2-kb *Sac*II–*Bam*HI genomic fragment was excised from pBluescript (pBS) bearing the 2-kb fragment at the *Sma*I site. pPGKBSD was obtained by replacing the puromycin-resistance gene in pPGKPuro (a gift from Dr. T. Yagi, National Institute for Physiological Sciences) (Watanabe *et al.*, 1995) with a blasticidin resistance gene from pMAM2-BSD (Kimura *et al.*, 1994). PGKneo, PGKpuro, PGKhyg, and PGKbsd cassettes from pPNT, pPGKPuro, pPGK-Hygro (a gift from Dr. A. Berns, The Netherlands Cancer Institute), and pPGKBSD, respectively, were cloned into blunt-ended *Hin*dIII sites of pBS and excised as *Xho*I–*Bam*HI fragments. Each of these fragments containing the PGK-driven drug resistance genes and the *Not*I–*Xho*I fragment of mouse *GAA1* described above were cloned into *Not*I–*Bam*HI sites of pPNT bearing the 7-kb fragment of mouse *GAA1* (see Figure 1A). F9 cells were electroporated with *Not*I-linearized targeting plasmids and selected 1 d later with appropriate drugs. Concentrations of G418, puromycin (Sigma, St. Louis, MO), hygromycin, and blasticidin were 380, 2, 500, and 4 μg/ml, respectively. Recombinants were screened by PCR with common 3′ primer and drug cassette-specific 5′ primers and were confirmed by Southern blotting using 1-kb *Eco*RV–*Eco*RI and 0.6-kb *Bam*HI–*Bam*HI genomic fragments as 5′ and 3′ probes, respectively (see Figure 1A).

Flow Cytometric Analysis

Cells were stained with biotinylated anti–Thy-1 G7 or anti-CD59 5H8 followed by phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA) and analyzed in a FACScan (Becton Dickinson, San Jose, CA) (Maeda *et al.*, 1998).

TLC Analysis of GPI Intermediates

GPI intermediates were metabolically radiolabeled with [^{3}H]mannose (American Radiolabeled Chemicals, St. Louis, MO) in the

presence of tunicamycin, extracted, and analyzed by TLC (Hirose *et al.*, 1992).

Isolation of Microsomal Membranes and In Vitro Translational Assay for GPI Attachment to Mini-Placental Alkaline Phosphatase

The amino acid sequence of mini-placental alkaline phosphatase (mini-PLAP) with Ser at the ω site, designed based on the reported sequence (Millan, 1986), was basically identical to that used by Udenfriend's group (Kodukula *et al.*, 1991), except for the presence of an additional five residues (Met-Leu-Gly-Pro-Cys) at the amino terminus. A coding region of this mini-PLAP was divided into three regions and amplified from human placental mRNA by RT-PCR and assembled in pSPUTK, an in vitro transcription vector (Stratagene, La Jolla, CA). Microsomal membranes were isolated basically according to a reported method (Maxwell *et al.*, 1995b; Chen *et al.*, 1996). The microsomal membranes were suspended in a suspension buffer containing 50 mM triethanolamine/250 mM sucrose, pH 7.5, at a determined concentration (50 OD units/ml at 280 nm in 1% SDS), frozen in liquid nitrogen, and stored at −80°C. Membranes of mutant and corresponding wild-type cells were prepared at the same time.

One microliter-capped mini-PLAP RNA (1 μg/μl) was translated at 30°C for 90 min in the following reaction mixture: 12.5 μl nuclease-treated rabbit reticulocyte lysate, 1 μl methionine-free amino acid mixture, 0.5 μl RNasin (all from Promega [Madison, WI]), 2 μl Redivue L-[^{35}S]methionine (Amersham, Arlington Heights, IL), 2.5 μl buffer composed of 100 mM potassium acetate, 4 mM magnesium acetate, 20 μg/ml each of antipain, aprotinin, bestatin, chymostatin, leupeptin, and pepstatin, 1.5 μl water, 4 μl microsomal membranes, and 1 μl water or 260 mM hydrazine. Reaction mixtures were diluted in 1 ml of a precipitation buffer consisting of 1% NP-40, 50 mM Tris, 150 mM NaCl, 0.025% sodium azide, and a complete protease inhibitor mixture tablet at the recommended concentration (Boehringer Mannheim, Mannheim, Germany), pH 7.8. Mini-PLAP proteins were precipitated with rabbit anti-PLAP antibody (Biomeda) and protein A-Sepharose, fractionated on a 15% SDS-PAGE gel, and visualized by BAS image analyzer (Fuji Photo Film, Tokyo, Japan).

Constructions and Functional Analyses of Chimeric GPI8*s*

To generate chimeric *GPI8*s, we divided human and yeast *GPI8* coding regions into four segments encoding an amino-terminal signal sequence (amino acids 1–42 of hGpi8p, 1–35 of yGpi8p), a highly conserved region (amino acids 43–304 of hGpi8p, 36–297 of yGpi8p), a nonconserved juxtatransmembrane region (amino acids 305–370 of hGpi8p, 298–384 of yGpi8p), and transmembrane cytoplasmic domains (amino acids 371–395 of hGpi8p, 385–411 of yGpi8p). Unique restriction enzyme sites were designed at boundaries of the regions. We amplified these segments by PCR and confirmed the sequences. All nucleotide sequences introduced to make restriction enzyme sites did not change amino acids. These segments were assembled in all possible combinations in pMEPyori. Chimeric *GPI8* cDNAs (2 μg) were lipofected into class K cells with DMRIE-C in Opti-MEM (Life Technologies, Gaithersburg, MD), and transfectants were analyzed for CD59 surface expression 2 d later.

Site-directed and Deletion Mutagenesis of GPI8s and Functional Assay

An *Eco*RI–*Nhe*I fragment of human *GPI8* and an *Eco*RI–*Bsp*119I fragment of yeast *GPI8* encoding a highly conserved region were excised from pMEPyori–hGPI8 and pMEPyori–yGPI8 and subcloned into pBS. We replaced codons of interest with alanine by means of oligonucleotide-directed mutagenesis. Mutated fragments were cloned back into original plasmids. Plasmids were transfected into class K cells by electroporation or by DMRIE-C. Restoration of CD59 expression was measured by flow cytometry. Deletion mutants of human *GPI8* were constructed by replacement of a *Nhe*I–*Not*I fragment of pMEPyori–hGPI8 with PCR-amplified shortened fragments.

Analysis of Protein Complexes

CHO cells (4×10^6) were electroporated with 15 μg each of plasmids at 960 μF and 250 V. Cells were grown in medium for 2 d to allow protein expression and then solubilized in 1 ml precipitation buffer (containing 1% NP-40) on ice for 1 h. We centrifuged the cell lysates at 18,000 × *g* for 20 min to remove cell debris and nuclei and then centrifuged the supernatants at 100,000 × *g* for 1 h to remove insoluble materials. The resulting cleared lysates were subjected to immunoprecipitation with anti-FLAG M2 beads (Eastman Kodak, Rochester, NY) or anti-HA (Roche, Mannheim, Germany) plus protein G beads (Pharmacia, Piscataway, NJ). After the first precipitation, the remaining supernatants were subjected to precipitation with glutathione beads (Pharmacia) or anti-FLAG M2 beads. These precipitates were washed in 1 ml precipitation buffer five times and analyzed by Western blotting as reported (Watanabe *et al.*, 1998).

RESULTS

Mammalian Gaa1p Is Essential for Attachment of GPI to Proteins

Human and mouse Gaa1ps have only 25% amino acid identity with *S. cerevisiae* Gaa1p (Hiroi *et al.*, 1998; Inoue *et al.*, 1999). To demonstrate that mammalian Gaa1p is involved in attachment of the GPI anchor, we disrupted mouse *GAA1* genes in F9 embryonal carcinoma cells by means of homologous recombination (Figure 1A). Perhaps because of an unexpected amplification of the *GAA1* gene during the disruption procedures, we needed to perform four homologous recombinations to eliminate all wild-type alleles of *GAA1* (Figure 1B). The *GAA1*-knockout F9 cells lost the surface expression of GPI-anchored proteins Thy-1 (Figure 2A, a and b), stem cell antigen-1, and heat stable antigen (our unpublished results). Their expression was restored after transfection of human *GAA1* cDNA (Figure 2A, c and d) and mouse *GAA1* cDNA (our unpublished results), indicating that mammalian *GAA1* is necessary for the cell surface expression of GPI-anchored proteins.

To confirm that biosynthesis of GPI is normal after disruption of *GAA1*, we metabolically labeled *GAA1*-knockout F9 cells with [^{3}H]mannose in the presence of tunicamycin and analyzed mannolipids by TLC. Mature forms of GPI termed H7 and H8 (Hirose *et al.*, 1992) were synthesized and accumulated in the *GAA1*-knockout cells (Figure 2B, lane 5) compared with the wild-type F9 cells (lane 6). This phenotype of *GAA1*-knockout cells was similar to that of *GPI8*-defective class K mutant cells that accumulate large amounts of H8 and H7 (lanes 3 and 4). These results together indicate that mammalian Gaa1p is not required for biosynthesis of GPI but is essential for attachment of GPI to proteins.

Gaa1p Is Required for the Cleavage of the GPI Attachment Signal Peptide

It was reported previously that a temporary carbonyl intermediate between the precursor protein and the GPI transamidase is formed during GPI-anchoring with a release of a cleaved

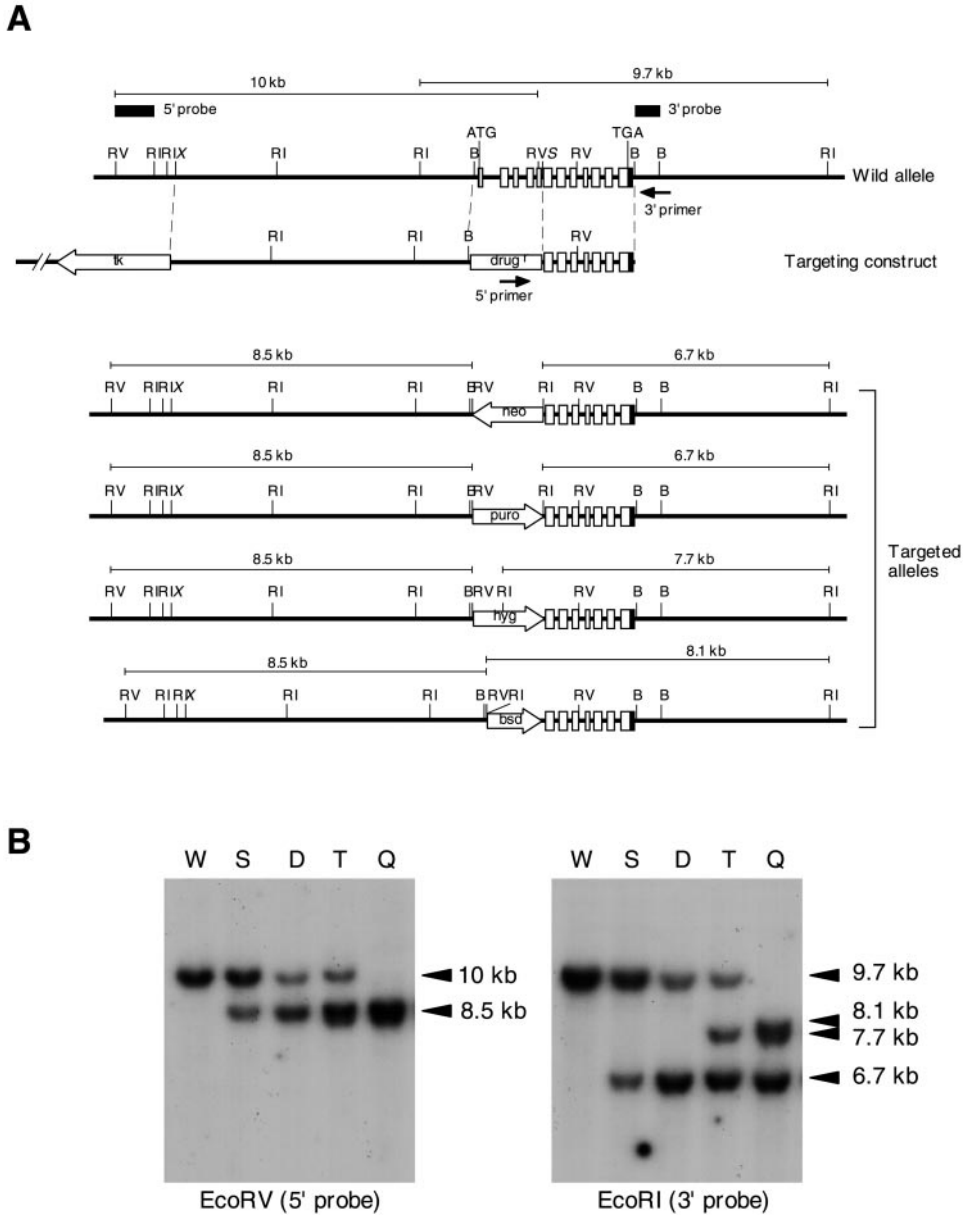

Figure 1. Generation of mouse *GAA1*-knockout cells. (A) Structure of the mouse *GAA1* gene (top), targeting constructs (middle), and expected disrupted alleles (bottom). Four consecutive recombination-targeting constructs in which drug resistance genes (drug^r) were neomycin (neo), puromycin (puro), hygromycin (hyg), and blasticidin (bsd) resistance genes, respectively, were used in this order. The mouse *GAA1* gene consists of 12 exons (shown in boxes: open areas, coding regions; closed areas, noncoding regions). Closed arrows represent PCR primers for screening recombinants. Probes for Southern analysis are indicated by thick bars. Predicted sizes of *Eco*RV- and *Eco*RI-digested fragments hybridized with 5′ and 3′ probes, respectively, are indicated above each allele. RV, *Eco*RV; RI, *Eco*RI; B, *Bam*HI; X, *Xba*I; S, *Sac*II (X and S are not unique). (B) Southern analysis of *GAA1*-targeted F9 cells. *Eco*RV-digested (left) and *Eco*RI-digested (right) genomic DNA from wild-type cells (W) and cells after single (S), double (D), triple (T), and quadruple (Q) recombinations were hybridized with the 5′ probe (left) and 3′ probe (right), respectively. The sizes of hybridized bands are indicated on the right.

signal peptide (Maxwell *et al.*, 1995a). We tested whether microsomal membranes of *GAA1*-knockout F9 cells can generate the carbonyl intermediate. We used a cell-free system (Kodukula *et al.*, 1991) in which a radiolabeled precursor protein, mini-PLAP, generated by in vitro transcription and translation is processed by the microsomal membranes bearing GPI transamidase. Microsomal membranes from wild-type CHO, EL4, K562, and F9 cells completed processing, generating the GPI-anchored form of mini-PLAP (Figure 3A, lanes 2, 4, 8, and 12) as well as pro–mini-PLAP (with a cleaved amino-terminal signal sequence). Membranes from CHO and F9 cells generated small amounts of free mini-PLAP (which lost the amino-terminal signal sequence as well as the GPI attachment signal peptide because of hydrolysis; lanes 2 and 12) (Maxwell *et al.*, 1995b), and membranes from K562 cells generated relatively more free mini-PLAP (lane 8). In the presence of hydrazine, generation of the GPI-anchored form was almost completely inhibited because of competition between GPI and excess hydrazine, resulting in generation of the hydrazide of free mini-PLAP (lanes 3, 5, 9, and 13). As reported previously, membranes from class F GPI synthesis mutant cells did not generate the GPI-anchored form because of a lack of mature GPI (lane 6) but formed the enzyme-substrate carbonyl intermediate, which was sensitive to hydrazine (lane 7) (Chen *et al.*, 1996). The membranes of class K cells did not generate the GPI-anchored form (lane 10) or the carbonyl intermediate (lane 11), as reported (Chen *et al.*, 1996). Similarly to class K cells, membranes from *GAA1*-knockout cells processed the amino-terminal signal peptide generating pro–mini-PLAP but did not generate the GPI-anchored form (lane 14) or the carbonyl intermediate

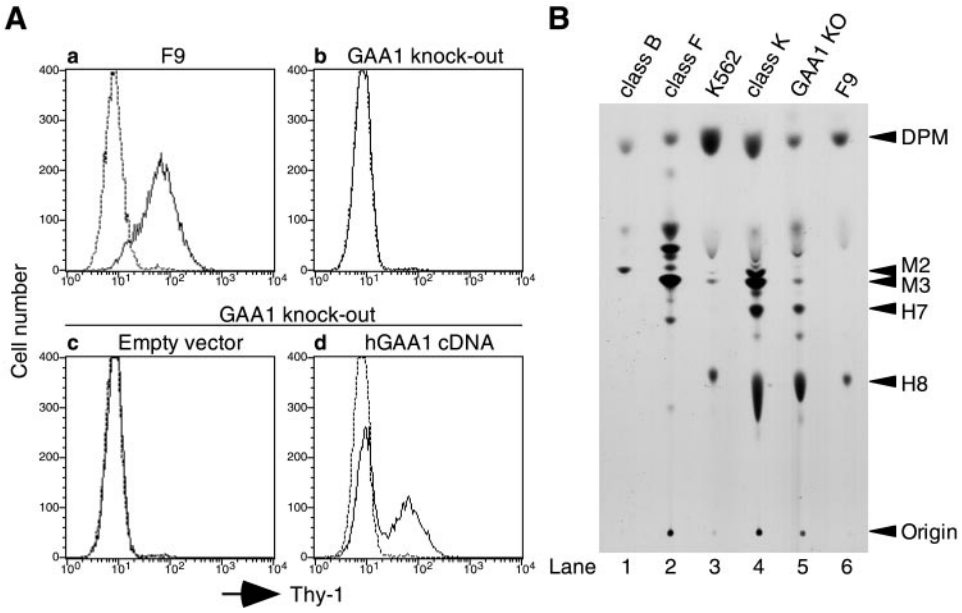

Figure 2. Characterization of *GAA1*-knockout cells. (A) Surface expression of GPI-anchored proteins. Parent F9 cells (a) and *GAA1*-knockout cells (b) were analyzed for the surface expression of Thy-1. *GAA1*-knockout cells transiently transfected with an empty vector (c) or human *GAA1* cDNA (d) were stained for Thy-1 2 d after transfection. Solid and dotted lines indicate staining with anti–Thy-1 antibody and that with secondary reagent alone, respectively. (B) Biosynthesis of GPI anchor intermediates analyzed by TLC. Cells were metabolically labeled with [^{3}H]mannose for 45 min in the presence of tunicamycin, and mannolipids were analyzed by TLC and fluorography. Identities of spots and origin are indicated on the right. DPM, dolichol–phosphate-mannose; M2 and M3, GPI intermediates bearing two and three mannose residues, respectively; H7 and H8, mature forms of GPI. Lanes 1 and 2, class B and F GPI-deficient mutants used to help identify the spots; lanes 3 and 4, wild-type and class K mutant of K562 cells; lanes 5 and 6, *GAA1*-knockout and wild-type F9 cells.

(lane 15). Generation of the GPI-anchored form was restored by transfection of human *GAA1* cDNA into *GAA1*-knockout cells (Figure 3B, lanes 6 and 7). Transfection of human *GPI8* cDNA had no effect (Figure 3B, lanes 8 and 9). These results indicate that Gaa1p acts before or during formation of the carbonyl intermediate.

Residues Conserved among Cysteine Proteases Are Essential for Gpi8p Activity

It has been suggested, based on sequence homology to cysteine proteases, that Gpi8p is the catalytic component of GPI transamidase that cleaves off the GPI attachment signal pep-

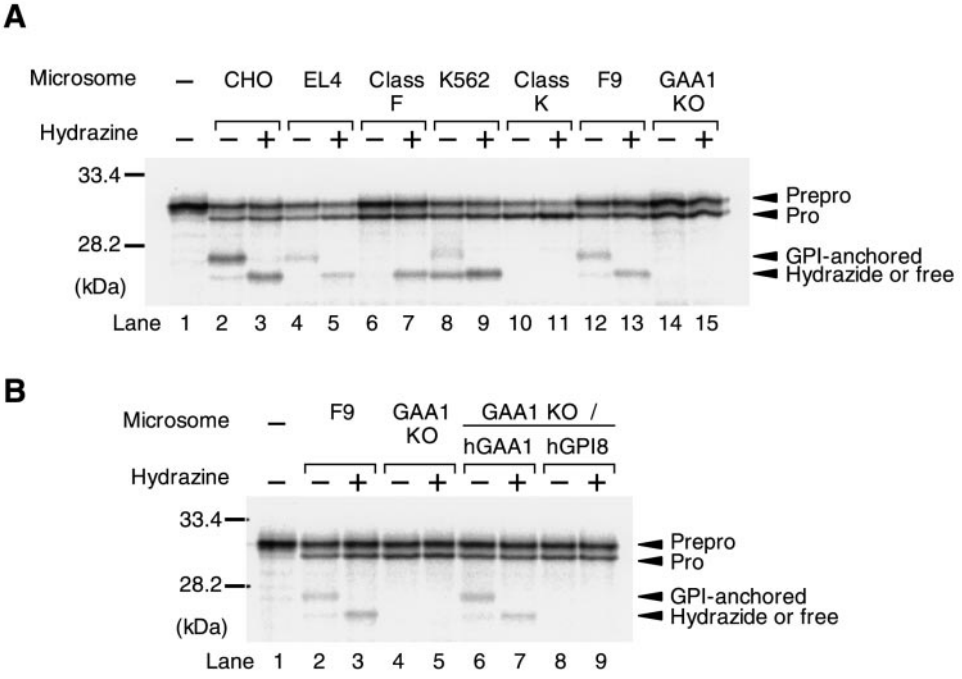

Figure 3. Microsomal membranes from *GAA1*-knockout cells do not form a carbonyl intermediate of mini-PLAP. Capped mini-PLAP mRNA was translated in vitro using rabbit reticulocyte lysate and microsomal membranes prepared from the indicated cells in the absence (−) or presence (+) of hydrazine. Lane 1 in both A and B shows a translation product in the absence of microsomal membranes, yielding prepro–mini-PLAP. Identities of other bands determined according to Kodukula *et al.* (1991) are shown on the right. Positions of prestained molecular markers are shown on the left. The cellular origin of the microsomal membranes is indicated as well as the presence or absence of hydrazine. In B, lanes 6 and 7 show the microsomal membranes from *GAA1*-knockout cells rescued with human *GAA1* cDNA, and lanes 8 and 9 show those from *GAA1*-knockout cells transfected with human *GPI8* cDNA.

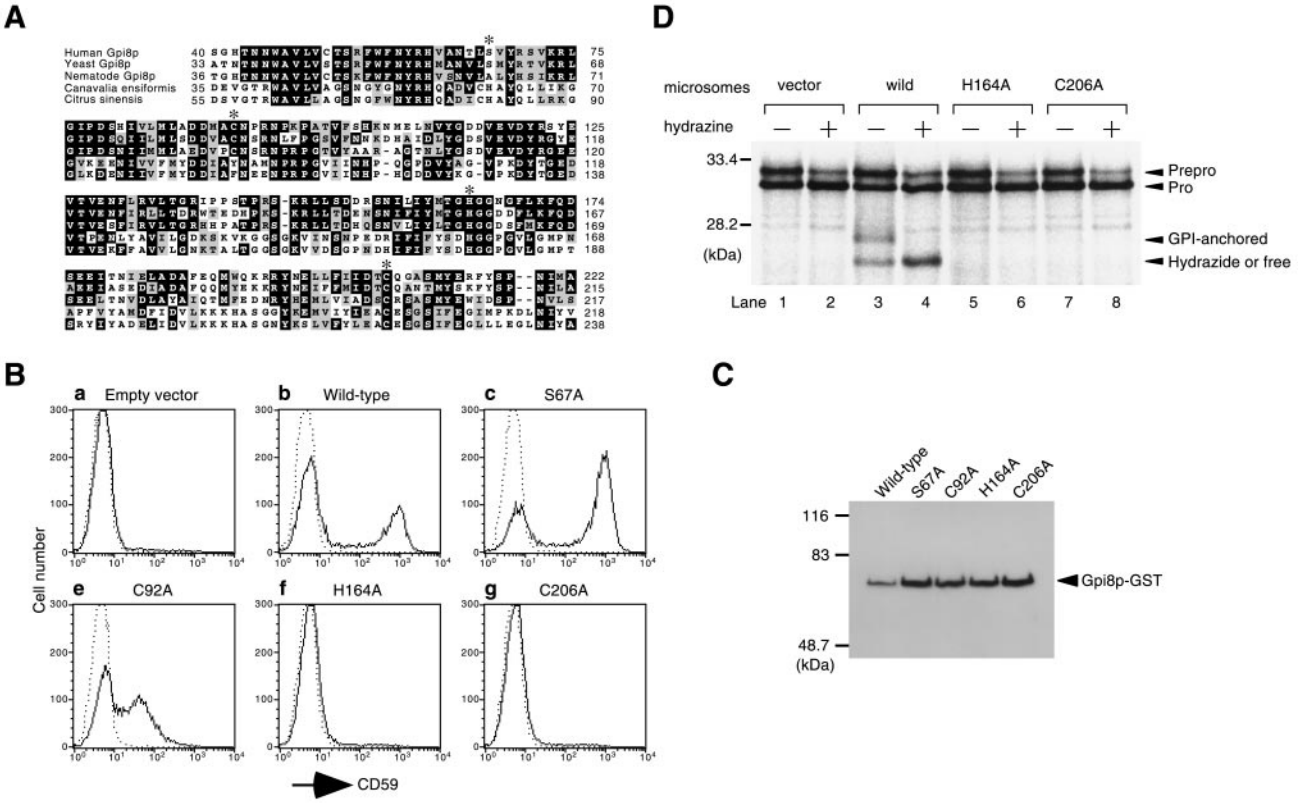

Figure 4. Cysteine and histidine residues conserved in Gpi8ps and cysteine proteases are essential for Gpi8p activity. (A) Alignment of partial sequences of human Gpi8p, yeast Gpi8p, nematode (*Caenorhabditis elegans*) Gpi8p (T05E11.6) (Wilson *et al.*, 1994), and two proteases belonging to a novel cysteine protease family from *Canavalia ensiformis* (Abe *et al.*, 1993), *Citrus sinensis* (Alonso and Granell, 1995) using Clustal W software. Amino acids of human Gpi8p substituted for alanine are marked with asterisks. (B) Activities of alanine mutants of human Gpi8p. GST-tagged wild-type and mutant human *GPI8* cDNAs were transiently transfected into class K cells. Cells were stained for CD59 and analyzed by flow cytometry 2 d after transfection. Solid and dotted lines indicate staining with anti-CD59 antibody and that with secondary reagent alone, respectively. (C) Expression of mutant Gpi8ps in the transfectants used in B. GST-tagged Gpi8ps in detergent extracts of the transfectants were collected with glutathione beads and analyzed by Western blotting with anti-GST antibody. Molecular markers in kilodaltons are indicated on the left. (D) In vitro assay with mini-PLAP. Microsomal membranes of class K cells stably expressing GST-tagged wild-type or mutant human Gpi8p were tested for the formation of carbonyl intermediates of mini-PLAP in vitro in a similar way as in Figure 3. Molecular markers in kilodaltons are indicated on the left.

tide (Benghezal *et al.*, 1996). To obtain experimental evidence for this, we mutagenized Cys206 and His164 in human Gpi8p, residues that are conserved in yeast and nematode Gpi8ps and members of a cysteine protease family and are likely to be involved in catalytic reaction (Figure 4A). We also mutagenized Cys92, which is conserved in Gpi8ps but not in other members of a cysteine protease family, and Ser67, which was suggested to be a possible active site (Benghezal *et al.*, 1996). As shown in Figure 4B, Ala substitutions of Cys206 and His164 resulted in complete loss of complementation of class K cells, whereas substitution of Cys92 only partially decreased the activity. Ser67 was not important. Expression of both Cys206Ala and His164Ala mutants was efficient as shown by Western blot analysis (Figure 4C).

To demonstrate that a lack of complementation of class K cells was due to a defect in cleavage of GPI attachment signals, we assayed transamidase activity using microsomal membranes of class K cells expressing these mutant Gpi8ps. The microsomes bearing these mutants produced no hydrazide form of mini-PLAP in the presence of hydrazine (Figure 4D, lanes 6 and 8). These results demonstrate that Cys206 and His164 are essential to form carbonyl intermediates.

We next tested whether corresponding cysteine and histidine in yeast Gpi8p (Cys199 and His157) are essential. Yeast Gpi8p had no activity when transfected into class K cells, maybe because of incompatibility with another mammalian component or components of GPI transamidase (our unpublished results). Therefore, we divided Gpi8ps into four regions, R1–R4 (Figure 5A), and constructed chimeras of yeast and human Gpi8ps. Replacement of R3, the least conserved intralumenal juxtamembrane region, of yeast Gpi8p with that of human origin rendered the chimeric protein functional in class K cells (our unpublished results), indicating that yeast R3 caused incompatibility and that other regions of yeast origin are interchangeable. To determine roles of Cys199 and His157 in yeast Gpi8p, we prepared respective Ala mutants using a chimera in which only

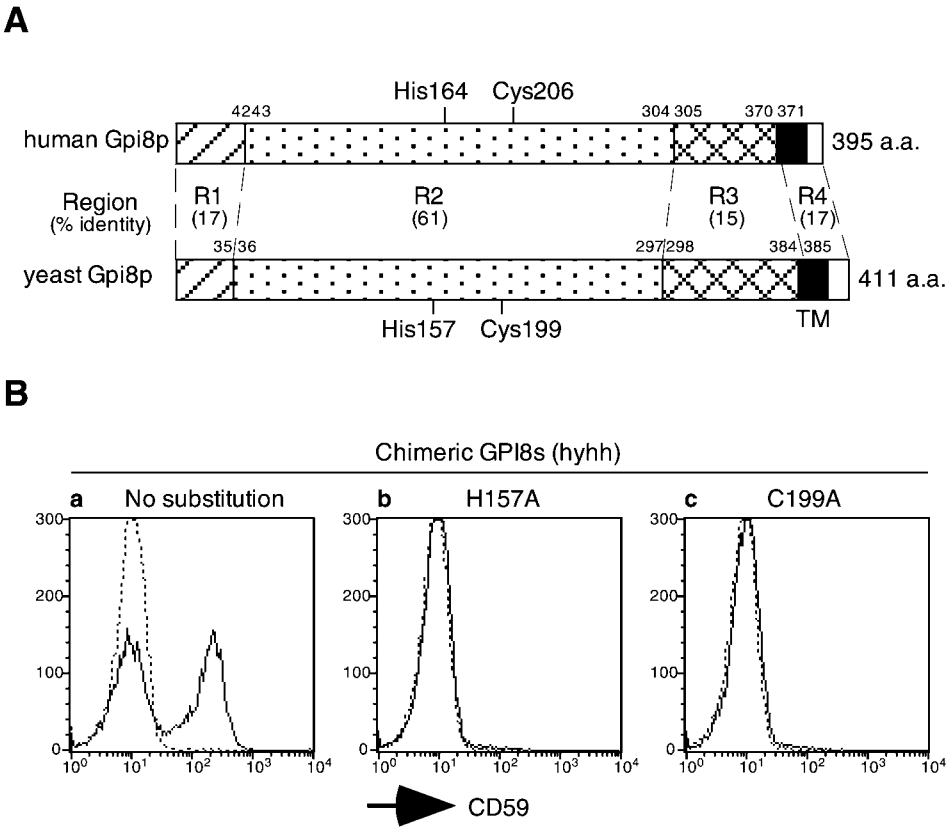

Figure 5. Conserved cysteine and histidine residues in yeast Gpi8p are essential for function. (A) Schematic representation of human and yeast Gpi8ps: hatched, dotted, cross-hatched, closed, and open boxes correspond to segments containing amino-terminal signal sequence, highly conserved, nonconserved, transmembrane (TM), and cytoplasmic regions, respectively. The two proteins were divided into four segments, R1–R4, and chimeric molecules were generated. The values in parentheses are percentage amino acid identity between the corresponding regions. Amino acid numbers and the positions of important histidine and cysteine residues are indicated. (B) Activities of a chimeric Gpi8p consisting of human R1, yeast R2, and human R3 and R4, and its alanine mutants. Chimeric Gpi8p and its mutants were transiently transfected into class K cells, and surface expression of CD59 was measured by flow cytometry. Solid and dotted lines indicate staining with anti-CD59 antibody and that with secondary reagent alone, respectively.

R2 was of yeast origin. Substitution of His157 or Cys199 with Ala showed complete loss of complementation activity (Figure 5B, b and c). Therefore, these conserved Cys and His are essential in both human and yeast Gpi8p for transamidase activity.

Gaa1p and Gpi8p Form a Protein Complex

The above results indicated that both Gaa1p and Gpi8p are components of the GPI transamidase. We next tested whether the two components form a complex. For this, we tagged Gaa1p and a control ER membrane protein ALDH (Masaki *et al.*, 1994) with the FLAG epitope, and Gpi8p and ALDH with GST. The tagged proteins were expressed in various combinations in CHO cells (Figure 6). FLAG-tagged Gaa1p or FLAG-tagged ALDH were immunoprecipitated with anti-FLAG beads from detergent extracts of the cells. The precipitates were analyzed by Western blotting with anti-GST (middle panel) and anti-FLAG (top panel) to assess coprecipitation. GST-tagged Gpi8p was coprecipitated with FLAG-tagged Gaa1p (lane 5), but not with FLAG-tagged ALDH (lane 4). GST-tagged ALDH was not coprecipitated with FLAG-tagged Gaa1p (lane 6), indicating a specific interaction between Gaa1p and Gpi8p. Analysis of the supernatant after immunoprecipitation with anti-FLAG beads by means of glutathione beads (bottom panel) demonstrated that more than half of GST-tagged Gpi8p was associated with FLAG-tagged Gaa1p (lanes 7 and 8).

Lumenal Region of Gpi8p Is Sufficient for Its Activity

Nematode Gpi8p contains only 322 amino acids and lacks a transmembrane domain. To determine the functional importance of a region of human Gpi8p including a transmembrane domain, we constructed mutants in which various lengths of the carboxyl-terminal portion were deleted (Figure 7A). Gpi8p mutant 321del bearing 321 amino acids and lacking the transmembrane domain retained its activity to complement class K mutant cells, indicating that the transmembrane domain is not necessary (Figure 7B, b). A mutant 310del bearing 310 amino acids did not have any activity (a). Therefore, a region from amino acids 311–321 is critical (see Figure 5A for location of this region and the transmembrane domain).

We next tested whether the loss of function of 310del was due to a lack of formation of a complex with Gaa1p. We analyzed complex formation between amino-terminally FLAG-tagged mutant Gpi8ps and HA-tagged Gaa1p by a coprecipitation assay (Figure 7C). Precipitates by anti-HA antibody were blotted with anti-HA (top panel) and anti-FLAG (middle panel) antibodies. FLAG-tagged wild-type Gpi8p was coprecipitated with HA-tagged Gaa1p (lane 12) but not with HA-tagged control protein ALDH (lane 7). FLAG-tagged ALDH was not coprecipitated with HA-tagged Gaa1p (lane 8), confirming the specificity of this assay. Deletion mutants 321del and 332del with class K

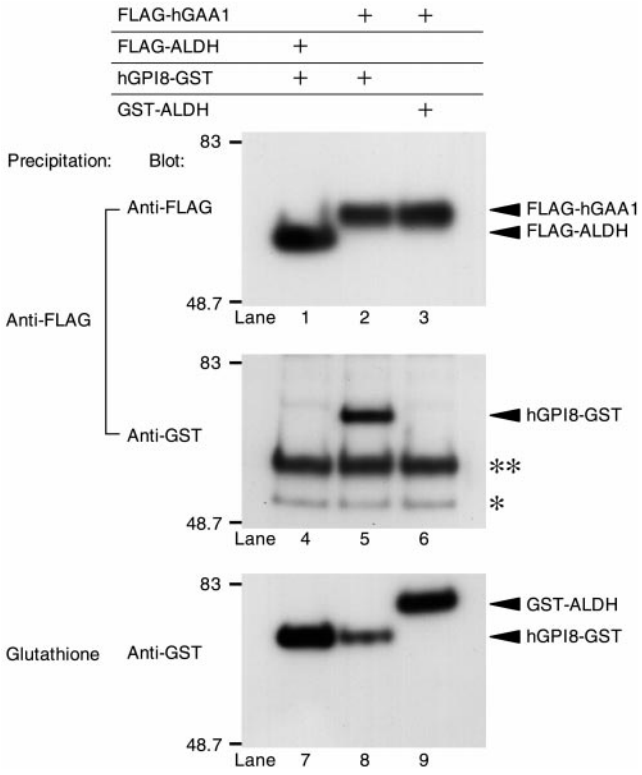

Figure 6. Association of human Gaa1p and Gpi8p shown by coimmunoprecipitation. Plasmids for FLAG- or GST-tagged proteins were transiently transfected into CHO cells in the indicated combinations. NP-40 extracts were prepared 2 d later and were first incubated for precipitation with anti-FLAG beads. The precipitates were analyzed by Western blotting with anti-FLAG (lanes 1–3) and anti-GST (lanes 4–6) antibodies. The supernatants of the first precipitation were then subjected to precipitation with glutathione beads and Western-blotted with anti-GST antibody (lanes 7–9). Double asterisks indicate the heavy chain of anti-FLAG antibody, and single asterisks indicate nonspecific bands of unknown origin (lanes 4–6). Molecular markers in kilodaltons are indicated on the left.

complementation activities formed complexes with Gaa1p (lanes 10 and 11). The nonfunctional mutant 310del also formed a complex with Gaa1p (lane 9), indicating that the transmembrane domain and amino acids 311–321 are not necessary for association with Gaa1p. Therefore, amino acids 311–321 must have another function that is essential for attachment of GPI.

DISCUSSION

A remarkable feature of the protein modification by GPI is that the carboxyl-terminal GPI attachment signal peptides do not have any consensus sequence but have only several rather nonstrict characteristics (Udenfriend and Kodukula, 1995). Nevertheless, they direct GPI-anchoring specifically. Characterization of the GPI transamidase that mediates this reaction is important for understanding the molecular mechanisms of GPI attachment.

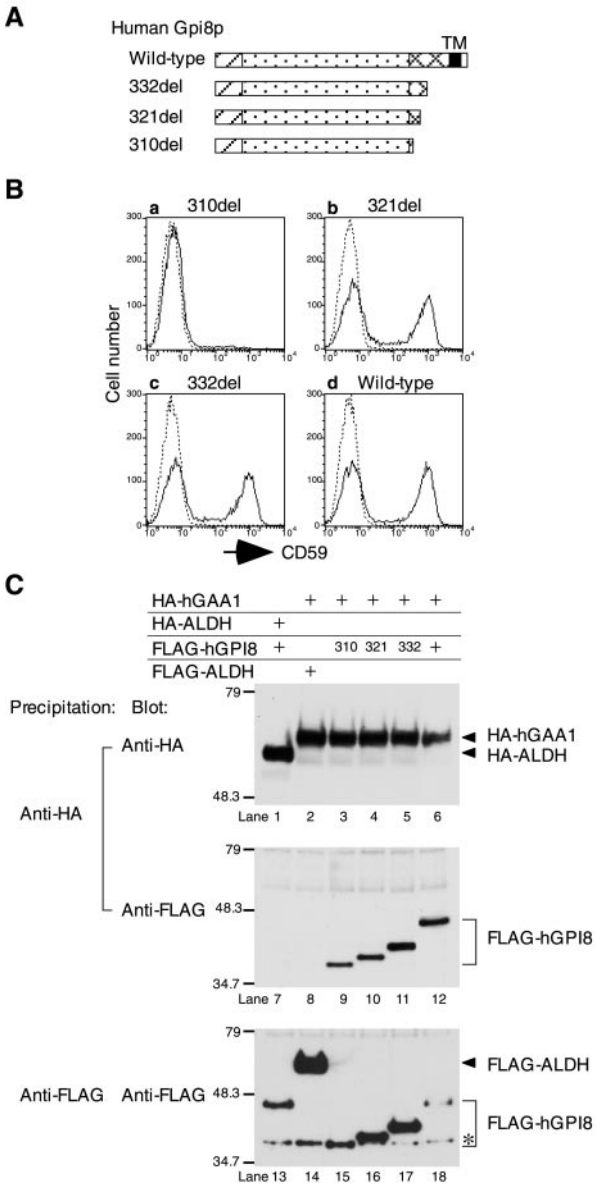

Figure 7. Lumenal portion of human Gpi8p is sufficient for its functions. (A) Schematic representation of wild-type and carboxyl-terminal deletion mutants of human Gpi8p. 310del, 321del, and 332del refer to deletion mutants of GPI8 encoding amino acids 1–310, 1–321, and 1–332, respectively. Segments are patterned in the same way as in Figure 5A. (B) Wild-type and deletion mutants of human GPI8 were transiently transfected into class K cells. Surface expression of CD59 was measured by flow cytometry 2 d later. Solid and dotted lines indicate staining with anti-CD59 antibody and that with secondary reagent alone, respectively. (C) Association of HA-tagged Gaa1p and FLAG-tagged deletion mutants of Gpi8p were analyzed by coprecipitation. Plasmids for HA- or FLAG-tagged proteins were transiently transfected into CHO cells in the indicated combinations. The precipitates from NP-40 lysates with anti-HA antibody were subjected to Western blotting with anti-HA (lanes 1–6) or anti-FLAG (lanes 7–12) antibodies. The remaining supernatants were then subjected to precipitation and blotted with anti-FLAG antibody (lanes 13–18). An asterisk indicates nonspecific bands of unknown origin that overlap with a band of 310del Gpi8p (lane 15). Molecular markers in kilodaltons are indicated on the left.

Gaa1p and Gpi8p Are Components of GPI Transamidase and Required for Generation of a Carbonyl Intermediate between Precursor Protein and the Transamidase

In the present study, we disrupted *GAA1* in murine F9 cells and found that Gaa1p is essential for GPI-anchoring of precursor proteins but not for GPI synthesis. In the absence of Gaa1p, a carbonyl intermediate between the precursor protein and the GPI transamidase was not formed. These characteristics of *GAA1*-disrupted cells are very similar to those of class K mutant cells that are defective in *GPI8* (Yu *et al.*, 1997). We also found that Gaa1p and Gpi8p formed a complex. Therefore, the two proteins are necessary for generation of the carbonyl intermediate.

Two steps are involved in generation of the carbonyl intermediate. First, the transamidase recognizes a GPI attachment signal peptide located at the carboxyl terminus of the precursor protein and presents it to the catalytic site. Second, the signal peptide should be cleaved by the catalytic site, resulting in formation of a carbonyl intermediate. It is known that GPI is not required for the transamidase to generate a carbonyl intermediate (Maxwell *et al.*, 1995a). Gpi8p should function in the second step because it has sequence homology to cysteine proteases and because its cysteine, which is conserved among members of the cysteine protease family, is essential for its function (Figures 4 and 5). It is not possible to predict functions of Gaa1p from its sequence because it has no significant homology to other proteins of known functions. Gaa1p could recognize the GPI attachment signal peptide. This possibility is supported by previous experiments that showed that overexpression of yeast *GAA1* could partially suppress the processing defect seen in the GPI signal peptide mutants of Gas1p (Hamburger *et al.*, 1995), or Gaa1p could act during formation of the carbonyl intermediate together with Gpi8p.

The GPI attachment signal peptide contains a carboxyl-terminal moderately hydrophobic region. It is thought to act as a temporary membrane anchorage of a precursor protein until it is recognized by the transamidase (Udenfriend and Kodukula, 1995). It was reported that substitution of valine for aspartic acid located within a hydrophobic region of GPI attachment signal of Qa-2 abolished GPI attachment and resulted in the expression as an integral transmembrane protein (Waneck *et al.*, 1988). It was also reported that the hydrophobic region is highly sensitive to substitution with charged residues (Nuoffer *et al.*, 1991; Yan *et al.*, 1998). These results indicate that moderate hydrophobicity is required for the GPI attachment signal and suggest that a hydrophobic domain of the transamidase would recognize it. Deletion mutants of human Gpi8p lacking a transmembrane region retained full activity to complement class K cells (Figure 7B), indicating that another component or components should be responsible. Both yeast and mammalian Gaa1ps have several hydrophobic regions (Hamburger *et al.*, 1995; Inoue *et al.*, 1999). Whether they are involved in this recognition is not clear at the moment.

Evidence That Gpi8p Has a Catalytic Site Involving Cysteine

We found that Cys206 and His164 of human Gpi8p and Cys199 and His157 of yeast Gpi8p are conserved in nematode Gpi8p, jack bean asparaginyl endopeptidase with transamidase activity and other members of a cysteine protease family (Figure 4A). Those residues were in fact essential for the function of human and yeast Gpi8ps (Figures 4, B and D, and 5B). Consistent with these results, this conserved His was predicted to be an important residue for a catalytic site of cysteine proteases (Alonso and Granell, 1995). The conserved Cys was not discussed in the same article, but instead another Cys (at the position of Leu66 of human Gpi8p) that is conserved in members of a cysteine protease family was speculated to be important (Alonso and Granell, 1995). The latter Cys, however, is not conserved in human and yeast Gpi8ps, and the same position is Leu in both Gpi8ps (Figure 4A). Based on this, Ser at the next position was alternatively predicted to be catalytic (Benghezal *et al.*, 1996); however, this Ser is not conserved in nematode Gpi8p, and indeed, Ser67 of human Gpi8p was not important (Figure 4B). Therefore, we conclude that cysteines homologous to Cys206 of human Gpi8p are essential for catalytic activities of members of this cysteine protease family.

Component That Recognizes and Presents GPI Glycolipid

The final step of GPI attachment would be a nucleophilic attack of the carbonyl intermediate between the precursor protein and Gpi8p by the terminal amino group of the preassembled GPI. Therefore, the GPI transamidase may contain a component that binds GPI. At the moment there is no information about this putative component. Gaa1p contains a large hydrophilic amino-terminal domain that would reside in the lumen of the ER (Hamburger *et al.*, 1995). This domain probably contains a binding site for Gpi8p but in addition could recognize GPI. Another possibility is the existence of a third component. Amino acids 311–321 of Gpi8p are essential for GPI attachment. This region is not necessary for association of Gpi8p with Gaa1p. It is unlikely that this region is involved in the catalytic reaction because there is no consensus amino acid in this region. Therefore, a likely role of this region is to associate with a third component. A genetic approach in yeast, isolating and characterizing mutants that are synthetic lethal with *gaa1–1*, may help to identify a putative third component. A biochemical approach, involving purification of the protein complex containing Gaa1p and Gpi8p, could also lead to identification of additional components.

ACKNOWLEDGMENTS

We thank Reika Watanabe for discussion and Keiko Kinoshita for technical assistance. This work was supported by grants from the Human Frontier Science Program and the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Abe, Y., Shirane, K., Yokosawa, H., Matsushita, H., Mitta, M., Kato, I., and Ishii, S.-I. (1993). Asparaginyl endopeptidase of jack bean seeds. J. Biol. Chem. *268*, 3525–3529.

Alonso, J.M., and Granell, A. (1995). A putative vacuolar processing protease is regulated by ethylene and also during fruit ripening in *Citrus* fruit. Plant Physiol. *109*, 541–547.

Benghezal, M., Benachour, A., Rusconi, S., Aebi, M., and Conzelmann, A. (1996). Yeast Gpi8p is essential for GPI anchor attachment onto proteins. EMBO J. *15*, 6575–6583.

Caro, L.H.P., Tettelin, H., Vossen, J.H., Ram, A.F.J., van den Ende, H., and Klis, F.M. (1997). *In silicio* identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. Yeast *13*, 1477–1489.

Chen, R., Udenfriend, S., Prince, G.M., Maxwell, S.E., Ramalingam, S., Gerber, L.D., Knez, J., and Medof, M.E. (1996). A defect in glycosylphosphatidylinositol (GPI) transamidase activity in mutant K cells is responsible for their inability to display GPI surface proteins. Proc. Natl. Acad. Sci. USA *93*, 2280–2284.

Furukawa, Y., Tsukamoto, K., and Ikezawa, H. (1997). Mutational analysis of the C-terminal signal peptide of bovine liver 5′-nucleotidase for GPI anchoring: a study on the significance of the hydrophilic spacer region. Biochim. Biophys. Acta *1328*, 185–196.

Gerber,L.D.,Kodukula,K.,andUdenfriend,S.(1992).Phosphatidylinositol glycan (PI-G) anchored membrane proteins. J. Biol. Chem. *267*, 12168–12173.

Hamburger, D., Egerton, M., and Riezman, H. (1995). Yeast Gaa1p is required for attachment of a completed GPI anchor onto proteins. J. Cell Biol. *129*, 629–639.

Hiroi, Y., Komuro, I., Chen, R., Hosoda, T., Mizuno, T., Kudoh, S., Georgescu, S.P., Medof, M.E., and Yazaki, Y. (1998). Molecular cloning of human homolog of yeast *GAA1* which is required for attachment of glycosylphosphatidylinositols to proteins. FEBS Lett. *421*, 252–258.

Hirose, S., Prince, G.M., Sevlever, D., Ravi, L., Rosenberry, T.L., Ueda, E., and Medof, M.E. (1992). Characterization of putative glycoinositol phospholipid anchor precursors in mammalian cells. Localization of phosphoethanolamine. J. Biol. Chem. *267*, 16968–16974.

Inoue, N., Ohishi, K., Endo, Y., Fujita, T., Takeda, J., and Kinoshita, T. (1999). Human and mouse GPAA1 (glycosylphosphatidylinositol anchor attachment 1) genes: genomic structures, chromosome loci and the presence of a minor class intron. Cytogenet. Cell Genet. *84*, 199–205.

Kawagoe, K., Kitamura, D., Okabe, M., Taniuchi, I., Ikawa, M., Watanabe, T., Kinoshita, T., and Takeda, J. (1996). GPI-anchor deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. Blood *87*, 3600–3606.

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S deaminase gene from *Aspergillus terreus* (BSD): a new drug resistance gene for transfection of mammalian cells. Biochim. Biophys. Acta *1219*, 653–659.

Kinoshita, T., Inoue, N., and Takeda, J. (1995). Defective glycosyl phosphatidylinositol anchor synthesis and paroxysmal nocturnal hemoglobinuria. Adv. Immunol. *60*, 57–103.

Kodukula, K., Micanovic, R., Gerber, L., Tamburrini, M., Brink, L., and Udenfriend, S. (1991). Biosynthesis of phosphatidylinositol glycan-anchored membrane proteins. J. Biol. Chem. *266*, 4464–4470.

Kollar, R., Reinhold, B.B., Petráková, E., Teh, H.J.C., Ashwell, G., Dragonová, J., Kapteyn, J.C., Klis, F.M., and Cabib, E. (1997). Architecture of the yeast cell wall. J. Biol. Chem. *272*, 17762–17775.

Leidich, S.D., Kostova, Z., Latek, R.R., Costello, L.C., Drapp, D.A., Gray, W., Fassler, J.S., and Orlean, P. (1995). Temperature-sensitive yeast GPI anchoring mutants gpi2 and gpi3 are defective in the synthesis of *N*-acetylglucosaminyl phosphatidylinositol: cloning of the GPI2 gene. J. Biol. Chem. *270*, 13029–13035.

Low, M.G. (1989). The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim. Biophys. Acta *988*, 427–454.

Lu, C.F., Montijn, R.C., Brown, J.L., Klis, F., Kurjan, J., Bussey, H., and Lipke, P.N. (1995). Glycosyl phosphatidylinositol-dependent cross-linking of alpha-agglutinin and beta-1,6-glucan in the *Saccharomyces cerevisiae* cell wall. J. Cell Biol. *128*, 333–340.

Maeda, Y., Tomita, S., Watanabe, R., Ohishi, K., and Kinoshita, T. (1998). DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate. EMBO J. *17*, 4920–4929.

Masaki, R., Yamamoto, A., and Tashiro, Y. (1994). Microsomal aldehyde dehydrogenase is localized to the endoplasmic reticulum via its carboxyl-terminal 35 amino acids. J. Cell Biol. *126*, 1407–1420.

Maxwell, S.E., Ramalingam, S., Gerber, L.D., Brink, L., and Sidney, U.(1995a).Anactivecarbonylformedduringglycosylphosphatidylinositol addition to a protein is evidence of catalysis by a transamidase. J. Biol. Chem. *270*, 19576–19582.

Maxwell, S.E., Ramalingam, S., Gerber, L.D., and Udenfriend, S. (1995b). Cleavage without anchor addition accompanies the processing of a nascent protein to its glycosylphosphatidylinositol-anchored form. Proc. Natl. Acad. Sci. USA *92*, 1550–1554.

Micanovic, R., Gerber, L.D., Berger, J., Kodukula, K., and Udenfriend, S. (1990). Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase. Proc. Natl. Acad. Sci. USA *87*, 157–161.

Millan, J.L. (1986). Molecular cloning and sequence analysis of human placental alkaline phosphatase. J. Biol. Chem. *1986*, 3112–3115.

Mohney, R.P., Knez, J.J., Ravi, L., Sevlever, D., Rosenberry, T.L., Hirose, S., and Medof, M.E. (1994). Glycoinositol phospholipid anchor-defective K562 mutants with biochemical lesions distinct from those in Thy-1- murine lymphoma mutants. J. Biol. Chem. *269*, 6536–6542.

Moran, P., Raab, H., Kohr, W.J., and Caras, I.W. (1991). Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site. J. Biol. Chem. *266*, 1250–1257.

Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A., and Takeda, J. (1999). Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system. Lab. Invest. *79*, 293–299.

Nuoffer, C., Horvath, A., and Riezman, H. (1993). Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein. J. Biol. Chem. *268*, 10558–10563.

Nuoffer, C., Jeno, P., Conzelmann, A., and Riezman, H. (1991). Determinants for glycophospholipid anchoring of the *Saccharomyces cerevisiae* GAS1 protein to the plasma membrane. Mol. Cell. Biol. *11*, 27–37.

Ohishi, K., Kurimoto, Y., Inoue, N., Endo, Y., Takeda, J., and Kinoshita, T. (1996). Cloning and characterization of the murine GPI anchor synthesis gene Pigf, a homologue of the human PIGF gene. Genomics *34*, 340–346.

Sharma, D.K., Vidugiriene, J., Bangs, J.D., and Menon, A.K. (1999). A cell-free assay for glycosylphosphatidylinositol anchoring in African trypanosomes. J. Biol. Chem. *274*, 16479–16486.

Takeda, J., and Kinoshita, T. (1995). GPI-anchor biosynthesis. Trends Biochem. Sci. *20*, 367–371.

Takeda, J., Miyata, T., Kawagoe, K., Iida, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani, T., and Kinoshita, T. (1993). Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell *73*, 703–711.

Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997). Tissue specific knock-out of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. Proc. Natl. Acad. Sci. USA *94*, 7400–7405.

Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. Cell *65*, 1153–1163.

Udenfriend, S., and Kodukula, K. (1995). How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu. Rev. Biochem. *64*, 563–591.

Waneck, G.L., Stein, M.E., and Flavell, R.A. (1988). Conversion of a PI-anchored protein to an integral membrane protein by a single amino acid mutation. Science *241*, 697–699.

Watanabe, R., Inoue, N., Westfall, B., Taron, C.H., Orlean, P., Takeda, J., and Kinoshita, T. (1998). The first step of glycosylphosphatidylinositol biosynthesis is mediated by a complex of PIG-A, PIG-H, PIG-C and GPI1. EMBO J. *17*, 877–885.

Watanabe, S., Kai, N., Yasuda, M., Kohmura, N., Sanbo, M., Mishina, M., and Yagi, T. (1995). Stable production of mutant mice from double gene converted ES cells with puromycin and neomycin. Biochem. Biophys. Res. Commun. *213*, 130–137.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., and Cooper, J. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature *368*, 32–38.

Yan, W., Shen, F., Dillon, B., and Ratnam, M. (1998). The hydrophobic domains in the carboxyl-terminal signal for GPI modification and in the amino-terminal leader peptide have similar structural requirements. J. Mol. Biol. *275*, 25–33.

Yu, J., Nagarajan, S., Knez, J.J., Udenfriend, S., Chen, R., and Medof, M.E. (1997). The affected gene underlying the class K glycosylphosphatidylinositol (GPI) surface protein defect codes for the GPI transamidase. Proc. Natl. Acad. Sci. USA *94*, 12580–12585.