Conversion of secretory proteins into membrane proteins by fusing with a glycosylphosphatidylinositol anchor signal of alkaline phosphatase

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Placental alkaline phosphatase (PLAP) is initially synthesized as a precursor (proPLAP) with a C-terminal extension. We constructed a recombinant cDNA which encodes a chimeric protein (α GL-PLAP) comprising rat α_{2u} -globulin (α GL) and the C-terminal extension of PLAP. Two molecular species (25 kDa and 22 kDa) were expressed in the COS-1 cell transfected with the cDNA for α GL-PLAP. Only the 22 kDa form was labelled with both [3 H]stearic acid and [3 H]ethanolamine. Upon digestion with phosphatidylinositol-specific phospholipase C the 22 kDa form was released into the medium, indicating that this form is anchored on the cell surface via glycosylphosphatidylinositol (GPI). A specific IgG raised against a C-terminal nonapeptide of proPLAP precipitated the 25 kDa form but not the 22 kDa form, suggesting that the 25 kDa form is a precursor retaining the C-terminal propeptide. When a mutant α GL-PLAP, in which the aspartic acid residue is replaced with tryptophan at a putative

cleavage/attachment site, was expressed in COS-1 cells, the 25 kDa precursor was the only form found inside the cell and retained in the endoplasmic reticulum, as judged by immunofluorescence microscopy. *In vitro* translation programmed with mRNAs coding for the wild-type and mutant forms of α GL-PLAP demonstrated that the C-terminal propeptide was cleaved from the wild-type chimeric protein, but not from the mutant one. This gave rise to the 22 kDa form attached with a GPI anchor, suggesting that GPI is covalently linked to the aspartic acid residue (Asp¹⁵⁹) of α GL-PLAP. Taken together, these results indicate that the C-terminal propeptide of PLAP functions as a signal to render α GL a GPI-linked membrane protein *in vitro* and *in vivo* in cultured cells, and that the chimeric protein constructed in this study may be useful for elucidating the mechanism underlying the cleavage of the propeptide and attachment of GPI, which occur in the endoplasmic reticulum.

INTRODUCTION

A number of membrane proteins are known to be anchored on the cell surface via glycosylphosphatidylinositol (GPI) [1–3]. It is generally assumed that GPI-linked proteins are initially synthesized as a precursor (proform) with a cleavable C-terminal extension which serves as a GPI anchor signal. A transamidase or transpeptidase is thought to catalyse the transfer of a pre-assembled GPI to the carboxyl group of a newly exposed C-terminal amino acid to form an amide linkage through the ethanolamine residue of GPI concomitant with cleavage of the C-terminal extension. Udenfriend's group [4,5] and our group [6,7] have unequivocally demonstrated that placental alkaline phosphatase (PLAP) is anchored on the cell surface via GPI. Chemical analysis of purified PLAP showed that the glycan moiety of GPI consists of ethanolamine, mannose, glucosamine and phosphate, while that the lipid moiety comprises palmitic acid, stearic acid (or myristic acid), inositol, glycerol and phosphate [5,7]. Incorporation of radiolabelled precursors into newly synthesized PLAP in cultured cells supports the data from chemical analysis [4,6]. Although the detailed structure has not been determined, available evidence suggests that the GPI anchor of PLAP has the same core structure of ethanolamine-glycan-phosphatidylinositol as those proposed for variant surface glycoprotein [8,9], Thy-1 [10,11] and erythrocyte acetylcholinesterase [12]. In these studies the cleavage/attachment site of GPI was assigned to an aspartic acid residue (Asp⁴⁸⁴) of PLAP [5,7]. Therefore, the cDNA sequence of PLAP [13] predicts that

the C-terminal extension, consisting of 29 amino acids (Ala⁴⁸⁵ to Pro⁵¹³), is cleaved from newly synthesized PLAP precursor (proPLAP) before the attachment of GPI. The importance of a stretch of hydrophobic amino acid residues in the prosequence for GPI attachment has been extensively studied [14,15]. During the course of biosynthetic studies of placenta-type alkaline phosphatase (hereafter also referred to as PLAP for convenience) using choriocarcinoma cells (JEG-3 cells) we found a short-lived intermediate of PLAP within the cell. This was considered to be proPLAP which still retained the C-terminal propeptide [6]. This was further confirmed by experiments with an antibody specific for a C-terminal nonapeptide of proPLAP [16], establishing that proPLAP is the immediate precursor of GPI-anchored PLAP, and that cleavage of the propeptide and attachment of GPI occur post-translationally in the JEG-3 cell. Interestingly, proPLAP was found to accumulate within the cell and never appeared on the cell surface under conditions where the acceptor GPI was depleted i.e. in the presence of 2-fluoro-2-deoxy-D-glucose (an inhibitor of the synthesis of dolichol phosphate-mannose [16]). This finding suggests that proPLAP is retained within the cell, although the exact subcellular location of the accumulated proPLAP remains unknown. Recently Moran and Caras [17] have reported that a fusion protein containing an uncleavable GPI anchor signal of decay-accelerating factor accumulated in a brefeldin A-sensitive compartment located between the endoplasmic reticulum (ER) and the Golgi apparatus, and have suggested that this salvage compartment might function as part of a system for quality control.

Abbreviations used: α GL, rat α_{2u} -globulin; ALB, rat serum albumin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; MEM, minimum essential medium; PLAP, placental alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C; proPLAP, PLAP precursor.

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In this study, we constructed two chimeric proteins containing the C-terminal extension of PLAP to see whether the propeptide acts as a GPI-anchor signal in cultured cells and in the translational system *in vitro*. We also engineered a chimeric protein with an uncleavable GPI signal by site-directed mutagenesis to examine the subcellular localization of the mutant chimeric protein.

MATERIALS AND METHODS

Materials

[³⁵S]Methionine (specific radioactivity > 800 Ci/mmol) and [9,10-³H]stearic acid (sp. radioactivity 21.5 Ci/mmol) were obtained from Dupont–New England Nuclear (Boston, MA, U.S.A.). [1-³H]Ethanolamine hydrochloride (sp. radioactivity 12.0 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL, U.S.A.); Protein A–Sepharose CL-4B from Pharmacia LKB Biotechnology (Uppsala, Sweden); Lipofectin Reagent from Gibco-BRL (Gaithersburg, MD, U.S.A.); pBluescriptSK⁻, pSG5 and mCAP mRNA capping kit from Stratagene (San Diego, CA, U.S.A.); pGEM3, rabbit reticulocyte lysates and pancreatic microsomes from Promega (Madison, WI, U.S.A.); Gene-Clean from Bio101 (La Jolla, CA, U.S.A.); rhodamine-conjugated goat anti-(rabbit IgG) Fab' from Cappel Laboratories (Malvern, PA, U.S.A.); aprotinin from Sigma Chemical Co. (St. Louis, MO, U.S.A.); antipain, chymostatin, elastatinal, leupeptin, pepstatin A and phosphoramidone from Protein Research Foundation (Osaka, Japan). Various DNA-modifying enzymes and restriction endonucleases were obtained from Nippon Gene (Tokyo, Japan) and Takara Shuzo (Kyoto, Japan). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from *Bacillus cereus* as described previously [18]. cDNA for human PLAP was isolated as described [13], and that of rat serum albumin (ALB) was obtained from Dr. Y. Kaneda (Osaka University, Osaka, Japan). Antisera against ALB [19], rat α_{2u} -globulin (α GL) [20] and human PLAP [6,21] were raised in rabbits as described previously. Antiserum against the decapeptide (Cys-Leu-Leu-Leu-Glu-Thr-Ala-Thr-Ala-Pro) corresponding to the C-terminal nonapeptide (Leu⁵⁰⁶–Pro⁵¹³) of proPLAP was prepared as described previously [16,22]. The specific IgG [anti-(C-peptide) IgG] was affinity purified on peptide-conjugated Affigel-10. The third component of complement (α chain, 115 kDa), serum albumin (68 kDa), α_1 -protease inhibitor (56 kDa), haptoglobin β -chain (36 kDa) and α GL (20 kDa), all secreted from [³⁵S]methionine-labelled rat hepatocytes, were immunisolated from the medium as described previously [23,24] and used as molecular standards. COS-1 cells were obtained from Dr. Mizuno (Japanese Cancer Research Resources Bank, Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum.

Construction of plasmids

A full-length cDNA for rat α GL was obtained by screening a λ Zap cDNA library constructed by Misumi et al. [25]. Using oligonucleotide-directed mutagenesis a silent single-base mutation (G to A) was introduced in order to destroy an internal *Eco*RI site of α GL cDNA in pBluescriptSK⁻. The α GL cDNA, which had been previously engineered to create an *Eco*RV site, was digested with *Eco*RV, giving rise to α GL cDNA lacking a sequence encoding the seven C-terminal amino acids (truncated α GL cDNA). pBluescriptSK⁻ containing ALB cDNA at the *Eco*RI site of the plasmid was digested with *Eco*RV, giving rise to cDNA lacking the sequence encoding the C-terminal 72 amino acids (truncated ALB cDNA). PLAP cDNA in the *Eco*RI site of pBluescriptSK⁻ was digested with *Nae*I and *Eco*RV sequentially.

The resulting 1.3 kb fragment, which encodes the C-terminal 33 amino acids of proPLAP, was separated by agarose gel electrophoresis and isolated using Gene-Clean. The fragment was blunt-end-ligated with predigested pBluescriptSK⁻ containing either the truncated α GL cDNA or truncated ALB cDNA. All recombinant DNAs were subcloned into pSG5 for expression, or into pGEM3 for *in vitro* transcription. Point mutations were made at desired sites according to the method described by Kunkel [26] using a Takara Mutan K kit (Takara Shuzo, Kyoto, Japan). Nucleotide sequences of all the constructs were verified by dideoxynucleotide sequencing using the Sequenase Version 2.0 kit (U.S. Biochemical Co., Cleveland, OH, U.S.A.).

Transfection

Transfection was carried out using Lipofectin Reagent essentially according to the manufacturer's protocol. Samples (5 μ g) of plasmid in 50 μ l of water were mixed with 10 μ g of Lipofectin in 50 μ l of water and the mixtures were incubated at room temperature for 15 min. After being diluted with 1.7 ml of serum-free DMEM, the plasmid–Lipofectin complex was added to a culture of COS-1 cells (at 30–50% confluency in a 60-mm diam. dish). After 6 h, the medium was removed and 4 ml of fresh DMEM supplemented with 10% (v/v) fetal-calf serum was added to each dish. The cells were incubated for 24–48 h before being used in the experiments.

Labelling cells and immunoprecipitation

Cells were preincubated with methionine-free minimum essential medium (MEM) for 30 min and incubated in fresh methionine-free MEM with 25–50 μ Ci of [³⁵S]methionine for 0.5–4 h. For labelling with [³H]stearic acid or [³H]ethanolamine, the cells were preincubated in MEM for 1 h and incubated in fresh MEM with 5 mCi of [³H]stearic acid or 1 mCi of [³H]ethanolamine for 8 h as described previously [6]. After the medium was removed, cells were lysed in 0.5 ml of lysis buffer [PBS containing 1% (v/v) Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS]. A protease inhibitor mixture (antipain, aprotinin, chymostatin, elastatinal, leupeptin, pepstatin A and phosphoramidone) was added to lysates and media (10 μ g of each/ml). The lysates were centrifuged at 15000 *g* for 10 min. The resultant supernatants were then subjected to immunoprecipitation as described previously [27]. The chimeric molecules expressed on the cell surface were selectively immunoprecipitated [16,28]. After being labelled with [³⁵S]methionine, the cells were rinsed with PBS and incubated with 2.0 ml of Dulbecco's PBS containing 3 μ l of anti- α GL serum at 4 °C for 1 h. After unbound antibodies were washed away with Dulbecco's PBS, the cells were further incubated with 10 μ g of purified α GL in 1 ml of Dulbecco's PBS for 1 h at 4 °C to mask the remaining antigen-binding sites. The cells were scraped and collected by centrifugation. To each pellet was added 0.5 ml of the lysis buffer. The antigen–antibody complex was isolated with Protein A–Sepharose. After being extensively washed, the immunocomplexes were boiled in Laemmli's sample buffer [29] and analysed by SDS/PAGE [12.5% (w/v) polyacrylamide gel], followed by fluorography as described previously [23].

PI-PLC treatment

Cells were washed with MEM and incubated in 1 ml of fresh MEM with or without PI-PLC (0.1 unit/ml) for 1 h at 37 °C in a humidified CO₂ incubator as described previously [6,16]. The cell lysate and medium were successfully subjected to immunoprecipitation as described above.

In vitro transcription/translation

Each plasmid was linearized with *Xba*I and collected by phenol-chloroform extraction, followed by ethanol precipitation. Transcription reactions were performed with an mCAP mRNA capping kit. *In vitro* translation reactions were performed in the Flexi rabbit reticulocyte lysate with [³⁵S]methionine at 30 °C for 90 min as described previously [30]. Where indicated, 1.8 equivalents of canine pancreatic microsomal membranes were added to a 25 µl sample of translation reaction mixture according to the manufacturer's protocol.

Phase separation

After *in vitro* translation, reticulocyte lysates were layered on a 0.3 ml cushion [0.5 M sucrose in 50 mM Tris/HCl buffer (pH 7.4) containing 25 mM KCl and 10 mM MgCl₂] and centrifuged at 230 000 *g*_{av} for 20 min in a Beckman TL-100 ultracentrifuge (TLA 100.2 rotor). The membrane pellets were suspended in 200 µl of 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 0.1 % of Triton X-114 and the protease inhibitor mixture (1 µg of each inhibitor/ml) and incubated with or without PI-PLC (0.05 unit) for 3 h at 37 °C. After incubation the suspensions were adjusted to a final concentration of 1 % Triton X-114 and subjected to phase separation according to the method of Bordier [31].

Immunofluorescence microscopy

COS-1 cells, grown on coverslips in a 35-mm diam. dish, were transfected with 2 µg of plasmid using 5 µg of Lipofectin as described above. After 24 h, the cells were fixed in 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature. The fixative was aspirated and the cells were rinsed with PBS. The cells were incubated further with PBS in the presence or absence of 0.1 % Triton X-100 for 20 min. Then intact cells and permeabilized cells were incubated with PBS containing 5 % (w/v) skimmed milk in the presence or absence of 0.1 % Triton X-100 at 4 °C overnight. The cells were incubated first with anti-αGL serum, and then with rhodamine-conjugated goat anti-(rabbit IgG) for 0.5–1 h. The coverslip was washed with PBS, mounted on a glass slide with 90 % (w/v) glycerol in PBS and observed microscopically.

RESULTS

Expression of PLAP and chimeric proteins in COS cells

Rat ALB (68 kDa) and αGL (20 kDa) are major plasma proteins synthesized in the liver and secreted into the blood. In contrast with ALB, αGL is rapidly discharged into the urine during circulation and as a result its concentration in the blood is kept relatively low [32]. When expressed in COS-1 cells, both ALB [33] and αGL (results not shown) are rapidly secreted into the medium. We constructed two recombinant cDNAs encoding ALB-PLAP and αGL-PLAP (which consist of ALB or αGL fused to a C-terminal extension of PLAP and transfected them into COS-1 cells. The cells transiently expressing PLAP, or each chimeric protein, were pulse-labelled for 30 min with [³⁵S]methionine, followed by a long chase interval to ensure that most of the newly synthesized secretory proteins were released into the medium. After 4 h of chase, PLAP and the chimeric proteins were immunoprecipitated from the cell lysates and medium. The immunoprecipitates were analysed by SDS/PAGE as shown in Figure 1. Both PLAP (lanes 2 and 3) and αGL-PLAP (lanes

6 and 7) were found to be associated with the cells, while a significant amount of newly synthesized ALB-PLAP molecule was secreted into the medium (lane 4). To determine whether cell-associated molecules are anchored on the cell surface via GPI, the transfected cells were incubated with or without PI-PLC, which is known to release alkaline phosphatase from the membranes [34,35]. As shown in Figure 2, both PLAP (lanes 1–4) and αGL-PLAP (lanes 9–12) were released into the medium by PI-PLC treatment. Significant amounts of the chimeric proteins, as well as PLAP, remained associated with the cell even after

Figure 1 Expression of PLAP and chimeric proteins in COS-1 cells

COS-1 cells, which had been transfected with each of the expression vectors pSG5-PLAP (lanes 2 and 3), pSG5-ALB (lanes 4 and 5) and pSG5-αGL-PLAP (lanes 6 and 7), were pulse-labelled with [³⁵S]methionine for 30 min and chased for 4 h in MEM. Both cell lysates (lanes 2, 4 and 6) and medium (lanes 3, 5 and 7) were subjected to immunoprecipitation with either anti-PLAP (lanes 2 and 3), anti-ALB (lanes 4 and 5) or anti-αGL (lanes 6 and 7). The immunocomplexes were analysed by SDS/PAGE, followed by fluorography. Molecular-mass standards are: the third component of complement (α chain, 115 kDa), ALB (68 kDa), α₁-protease inhibitor (56 kDa), haptoglobin β chain (36 kDa) and αGL (20 kDa) from the top (lane 1).

Figure 2 Release of cell-surface-associated molecules by PI-PLC

COS-1 cells transfected with pSG5-PLAP (lanes 1–4), pSG5-ALB-PLAP (lane 5–8) or pSG5-αGL-PLAP (lanes 9–12) were labelled with [³⁵S]methionine for 2 h. The cells were washed with MEM and further incubated in 1 ml of MEM in the presence (lanes 2, 4, 6, 8, 10 and 12) or absence (lanes 1, 3, 5, 7, 9 and 11) of PI-PLC for 1 h at 37 °C. After incubation, both cell lysates (lanes 1, 2, 5, 6, 9 and 10) and medium (lanes 3, 4, 7, 8, 11 and 12) were subjected to immunoprecipitation, followed by SDS/PAGE and fluorography, as shown in Figure 1.

Figure 3 Schematic diagram showing the fusion protein α GL-PLAP

(a) An amino acid sequence of the C-terminal region of pro- α GL-PLAP using the single-letter code. The amino acids derived from α GL and those from PLAP are shown by lower- and upper-case letters respectively. Position numbers above the original sequence are also shown for α GL (above the sequence) and for PLAP (below the sequence). The thick arrow indicates a predicted cleavage site. The cleavage of the propeptide exposes a new C-terminal amino acid (Asp¹⁵⁹), which is assumed to be modified by GPI. Anti-(C-peptide) IgG was raised against the C-terminal nonapeptide (thick bar) of proPLAP. (b) Structures of precursors and mature forms of α GL-PLAP deduced from the recombinant cDNA sequence for α GL-PLAP and their reactivities with anti- α GL and anti-(C-peptide) antibodies.

Figure 4 Analysis of the expressed chimeric protein for identification of the pro- and mature forms

COS-1 cells transfected with pSG5- α GL-PLAP were labelled with [³⁵S]methionine for 3 h (lanes 1–5) or [³H]ethanolamine for 8 h (lanes 6 and 7). Cells were lysed immediately (lanes 1 and 4) or further incubated with PI-PLC (lanes 2, 3, 6 and 7). The cell lysates (lanes 1, 2 and 6) and media (lanes 3 and 7) were immunoprecipitated with anti- α GL. The cell lysate (lane 4) was immunoprecipitated using anti-(C-peptide) IgG. The protein expressed on the cell surface (lane 5) was selectively immunoprecipitated with anti- α GL as described in the Materials and methods section.

digestion with PI-PLC. Similar findings were reported by others [4,36]. As PLAP is almost completely released from COS cells in suspension [4], it is likely that PI-PLC is not able to gain access

Figure 5 Expression of a mutant chimeric protein in COS cells

COS-1 cells expressing wild-type α GL-PLAP (lanes 1, 2 and 5) or mutant α GL-PLAP (lanes 3, 4 and 6) were labelled with [³⁵S]methionine for 3 h (lanes 1–4) or with [³H]stearic acid (lanes 5 and 6) for 8 h. Cells were lysed before (lanes 5 and 6) or after (lanes 1–4) PI-PLC treatment. The cell lysates (lanes 1, 3, 5 and 6) and media (lanes 2 and 4) were subjected to immunoprecipitation with anti- α GL. The immunoprecipitates were analysed by SDS/PAGE and fluorography.

to PLAP molecules on the cell surface facing the substratum. The cell-associated ALB-PLAP was also susceptible to PI-PLC (Figure 2; lanes 5–8). Because of incompleteness of GPI anchoring, we did not examine the ALB-PLAP chimeric protein further.

The recombinant cDNA for α GL-PLAP encodes a prepro- α GL-PLAP consisting of a signal sequence for α GL (19 amino acids), truncated mature α GL (155 amino acids) and the C-terminal sequence (33 amino acids) derived from proPLAP arrayed in tandem (Figure 3). Proteolytic cleavages of the N-terminal signal sequence and the C-terminal propeptide were analysed by immunoprecipitation using anti- α GL or anti-(C-peptide) IgG (which specifically recognizes the C-terminal nonapeptide of proPLAP [16]), followed by SDS/PAGE. Modification of the chimeric protein with GPI was examined by treatment with PI-PLC with or without phase separation using Triton X-114. Two molecular forms of α GL-PLAP (25 kDa and 22 kDa) were consistently observed in the transfected cells, as shown in Figure 4 (lane 1). Anti-(C-peptide) IgG precipitated the 25 kDa form only (lane 4), indicating that this form retains the C-terminal propeptide. On the other hand only the 22 kDa form appeared on the cell surface, as shown by cell-surface immunoprecipitation (lane 5). Incorporation of [³H]ethanolamine into the 22 kDa form (lane 6) further substantiated the hypothesis that this molecular species is modified with GPI. Thus it seems likely that the 25 kDa form is a precursor (proform) which is converted into the 22 kDa form upon cleavage of the C-terminal propeptide and the addition of GPI. However, the 25 kDa form was not completely converted into the 22 kDa form even after a prolonged time of chase, indicating that a limited amount of

Figure 6 Detection of the expressed proteins by immunofluorescence

COS-1 cells expressing wild-type α GL-PLAP (panels A and B) or mutant α GL-PLAP (panels C and D) were fixed and incubated with anti- α GL in the presence (B and D) or absence (A and C) of 0.1% Triton X-100, followed by rhodamine-conjugated anti-(rabbit IgG).

the precursor overproduced in the transfected cells is properly processed into the mature 22 kDa form. Removal of the phosphatidylinositol moiety from the 22 kDa form by PI-PLC resulted in a slightly reduced mobility on the polyacrylamide gel (Figure 4, lanes 6 and 7).

Mutation of the cleavage/attachment site of GPI

Mature PLAP has been shown to be covalently linked to GPI at an aspartic acid residue (Asp⁴⁸⁴) which is located 30 residues N-terminal to proline at the C-terminus of proPLAP (Figure 3) [5,7]. To see if the same aspartic acid residue is attached to GPI, site-specific mutagenesis was performed to substitute the aspartic acid residue with tryptophan at a putative cleavage/attachment site (Asp¹⁵⁹) of α GL-PLAP. Micanovic et al. [37,38] have reported that PLAP, in which aspartic acid at the cleavage/attachment site is replaced with tryptophan, failed to acquire the GPI anchor. Figure 5 shows that the 22 kDa form was not found in the cells expressing mutant α GL-PLAP (lane 3), indicating that the substitution of aspartic acid (Asp¹⁵⁹) with tryptophan abolishes the cleavage of propeptide. [³H]Stearate was incorporated into wild-type α GL-PLAP (lane 5), but not into the mutant chimeric protein (lane 6), indicating that the attachment of GPI is also impaired. Furthermore the mutant chimeric protein was totally resistant to PI-PLC digestion (Figure 5, lane 4). Compatible with this finding, cell-surface immunofluorescence staining revealed that the mutant α GL-PLAP did not appear on the cell surface (Figure 6, panel C). Permeabilized cells expressing

mutant chimeric protein displayed a reticular network pattern of fluorescence characteristic of ER localization, in addition to staining of the nuclear envelope (Figure 6, panel D). As the mutant chimeric protein was not secreted into the medium (results not shown), these results indicate that the chimeric protein with the uncleavable GPI-anchor signal does not undergo modification by GPI and is retained within the ER.

In vitro transcription/translation

A single 28 kDa product was synthesized when mRNA for α GL-PLAP was translated *in vitro* (Figure 7, lane 1), presumably representing a preproform (Figure 3). The 28 kDa form disappeared when translated in the presence of microsomes (Figure 7, lane 3), compatible with the notion that the signal sequence is cleaved off during translocation of the chimeric molecule across the microsome membrane as in the case of α GL [20]. Instead, there appeared to be two bands, one of which co-migrated on the SDS/polyacrylamide gel with the 25 kDa form found in the transfected COS cell (Figure 7, lanes 3 and 9), suggesting that this form represents pro- α GL-PLAP. This was confirmed by immunoprecipitation with anti-(C-peptide) IgG (Figure 7, lane 4). The other band (22 kDa) was not precipitable with anti-(C-peptide) IgG (Figure 7, lanes 3 and 4), indicating that the propeptide had been removed from this species. The 22 kDa form was not produced when the mRNA for the mutant chimeric protein was translated in the presence of microsomes (Figure 7, lane 7). Thus, consistent with the results with transfected cells

Figure 7 *In vitro* transcription and translation of α GL-PLAP

Translation assays, programmed with mRNAs coding for wild-type α GL-PLAP (lanes 1–4) and mutant α GL-PLAP (lanes 5–8), were performed in the presence (lanes 3, 4, 7 and 8) or absence (lanes 1, 2, 5 and 6) of microsomes. Reaction mixtures were subjected to immunoprecipitation with anti- α GL (lanes 1, 3, 5 and 7) or anti-(C-peptide) IgG (lanes 2, 4, 6 and 8). The immunoprecipitates were analysed by SDS/PAGE and fluorography. α GL-PLAP expressed in COS-1 cells was also immunoprecipitated from the cell lysate (lane 9) and medium (lane 10) after PI-PLC treatment as described in Figure 4. A band marked by (·) is a contaminant occasionally observed during immunoprecipitations.

Figure 8 Phase separation of *in vitro* translated products

The wild-type α GL-PLAP (lanes 1–4) or mutant α GL-PLAP (lanes 5–8) were translated in the presence of microsomes as described in Figure 7. The products were subjected to phase distribution into Triton X-114 (de) and water (aq) before (lanes 1, 2, 5 and 6) or after (lanes 3, 4, 7 and 8) PI-PLC treatment. A band marked by (·) is a contaminant occasionally observed during immunoprecipitations.

(Figure 5), the mutant chimeric protein failed to undergo cleavage of the C-terminal propeptide *in vitro*.

To determine whether the 22 kDa form synthesized *in vitro* is GPI-linked, the translation products were subjected to phase separation into Triton X-114 and water fractions, as shown in

Figure 8. More than 60% of the 22 kDa species formed *in vitro* was recovered in the detergent phase (Figure 8, lanes 1 and 2). This is in contrast with the behaviour of the 25 kDa form, which was completely separated into the aqueous phase. Furthermore, after PI-PLC treatment the 22 kDa form was found to be largely distributed into the aqueous phase (Figure 8, lanes 3 and 4), strongly indicating that the 22 kDa form synthesized *in vitro* is attached partly, if not completely, by GPI. The reason why some of the 22 kDa molecules in the detergent phase were resistant to PI-PLC remains unknown (Figure 8, lanes 1 and 3). This could occur if not all of the 22 kDa species was able to gain access to PI-PLC under conditions where the digestion was performed. However, we cannot rule out the possibility that some of the GPI-linked 22 kDa species formed *in vitro* are inherently resistant to PI-PLC, due to acylation of the inositol moiety.

DISCUSSION

As a model of GPI-anchored proteins we have constructed two recombinant cDNAs encoding either ALB or α GL fused to the GPI anchor signal (the C-terminal 33 amino acids) of proPLAP. The rationale of these constructions is that both plasma proteins are non-glycosylated and hence mobility shifts due to the sequential proteolytic cleavages of the N-terminal presequence and C-terminal prosequence, which occur before and concomitant with the attachment of GPI, are not obscured by the acquisition and modifications of N-linked oligosaccharide chains when analysed by SDS/PAGE (Figure 3). There is a precedent to this study. Kodukula et al. [39] studied the synthesis and processing of miniPLAP in which 60% of the internal coding sequence of PLAP was deleted. Both the N-terminal signal sequence and the C-terminal prosequence of miniPLAP were found to be efficiently cleaved in the COS cell and the *in vitro* translation system supplemented with microsomes, although miniPLAP failed to appear on the cell surface [39].

A single 28 kDa protein was produced by the *in vitro* translation system programmed with mRNA coding for α GL-PLAP, which presumably represents a prepro- α GL-PLAP (Figure 7, lane 1). The presequence was almost completely cleaved when translated in the presence of microsomes, giving rise to the 25 and 22 kDa forms (Figure 7, lane 3). The 25 kDa form is assumed to be the proform retaining the C-terminal propeptide as this form, but not the 22 kDa form, is precipitable with anti-(C-peptide) IgG (Figure 7, lane 4). Triton X-114 partitioning, in combination with PI-PLC digestion, definitely showed that the 22 kDa form produced *in vitro* is partly, if not completely, GPI-linked (Figure 8, lanes 1–4). The two molecular species of α GL-PLAP were also found in COS-1 cells transiently expressing α GL-PLAP. Only the 22 kDa form was covalently attached by GPI. This was concluded from the following evidence: first, ethanolamine (Figure 4) and stearic acid (Figure 5), both of which are components of the GPI moiety of PLAP [6], were incorporated into the 22 kDa form. Secondly, this form was found to be released into the medium upon digestion with PI-PLC (Figure 4). The cleavage/attachment site of α GL-PLAP was indirectly estimated. Replacement of aspartic acid with tryptophan by site-directed mutagenesis abolished cleavage of the propeptide and attachment by GPI, both *in vivo* in cultured cells (Figure 5) and *in vitro* (Figure 7), as reported with regard to PLAP [37,38] and miniPLAP [39,40]. This suggests that the 22 kDa of a α GL-PLAP was modified by GPI at the aspartic acid residue (Asp¹⁶⁹) of the chimeric protein. Thus the GPI-anchoring signal of PLAP alone is sufficient to convert α GL, a soluble secretory protein, into the membrane-bound form tailed with GPI.

In contrast with the 22 kDa form attached to GPI, the 25 kDa proform never appeared on the cell surface. Three lines of evidence support this notion. First, cell-surface immunoprecipitation failed to detect the 25 kDa form (Figure 4). Secondly, the 25 kDa proform was not released into the medium from the cell surface upon digestion with PI-PLC under conditions where the 22 kDa form was released (Figure 4). Thirdly, the mutant 25 kDa chimeric protein was found not to be transported to the cell surface, as judged by immunofluorescence microscopy (Figure 6, panel C). Rather the 25 kDa form with the uncleavable GPI anchor signal was localized in the ER, as revealed by immunofluorescent staining of permeabilized cells. A similar observation was also made in the permeabilized cells expressing wild-type chimeric protein (Figure 6, panel B), where the 25 kDa form bearing the uncleaved GPI signal was a major product (Figure 4). In accord with our findings, Micanovic et al. [37] reported that PLAP with an uncleavable GPI signal was retained in the ER. These findings raise the possibility that both the uncleaved and uncleavable GPI-anchor signal of PLAP serve as sorts of retention signals for the chimeric protein and PLAP to be localized in the ER. Furthermore, Delahunty et al. [41] have quite recently reported that a GPI-anchoring signal of Q7^b functions as an ER retention signal, possibly by causing Q7^b to form a multimeric complex. However, not all the precursors with uncleaved GPI anchor signals are retained within the ER. Some precursors are known to leave the ER and reside in the ER-Golgi intermediate compartment [17,42], the Golgi complex [42,43] or lysosomes [44], indicating that the fate and subcellular localization of precursors with uncleaved GPI-anchoring signals may be different from one precursor to another.

As in the case of α GL, the GPI-anchoring signal of PLAP was found to be able to direct the attachment of GPI to ALB; however, a significant amount of the fusion protein ALB-PLAP was secreted into the medium (Figures 1 and 2). The reason of this leakage is not known at present. The secreted form of ALB-PLAP migrated to the same position as the cell-associated GPI-linked one on the SDS/polyacrylamide gel (Figure 1), implying that the C-terminal propeptide had been cleaved from the secretory form before discharge into the medium. This possibility is currently being examined.

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