Biosynthesis of phosphatidylinositol-glycan (PI-G)-anchored membrane proteins in cell-free systems: Cleavage of the nascent protein and addition of the PI-G moiety depend on the size of the COOH-terminal signal peptide

(COOH-terminal processing/transamidase/truncation mutants)

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ABSTRACT Nascent translation products of PI-G-anchored membrane proteins contain both NH₂- and COOH-terminal signal sequences of ≈15–30 residues that are removed during processing. Removal of the latter occurs concomitant with the addition of the PI-G moiety to the newly formed COOH terminus. In human placental alkaline phosphatase (PLAP) the COOH-terminal signal peptide contains 29 residues. An engineered form of PLAP, miniPLAP 208, containing the same NH₂- and COOH-terminal signal peptides as PLAP, was used as a substrate for cell-free processing. A comparison was made with mutants (Δ202, Δ197, Δ184, and Δ179) truncated at the COOH terminus. Intact preprominiPLAP 208 and truncated Δ202 were processed to yield the same mature product which, by size and distribution between Triton X-114 and water before and after treatment with inositol-specific phospholipases, indicates that it contained the PI-G moiety. Mutants that were further truncated at the COOH terminus, miniPLAPs Δ197, Δ184, and Δ179, were processed only at their NH₂ termini. Those portions of the COOH-terminal sequence in miniPLAPs Δ197 and Δ184 that extended beyond residue 179 were not removed during processing.

It is generally recognized that nascent proteins destined to be processed to a PI-G-anchored membrane form contain a hydrophobic signal peptide at both their NH₂ and COOH termini (1). Studies carried out in intact cells transfected with mutant cDNAs indicate that the COOH-terminal peptide must be of a certain minimal size and hydrophobicity for cleavage and PI-G tailing to occur (2). The COOH terminus shown in Fig. 1 (top sequence, residues 179–208) is the same as in the prepro form of wild-type human placental alkaline phosphatase (PLAP). The truncated mutants Δ202, Δ197, and Δ184 correspond to mutants 507, 502, and 489 of wild-type PLAP that were investigated previously (2). In those studies, carried out in intact cells, it was found that removal of six residues from the COOH terminus of preproPLAP still permitted processing to a membrane-bound, PI-G-tailed form. However, further truncation yielded secreted forms of PLAP that were not PI-G tailed.

What could not be determined from the earlier experiments with PLAP in intact cells is whether the truncated forms that were not PI-G tailed were also cleaved at Asp-484 prior to secretion or were secreted without further processing at their COOH termini. The large size of PLAP and the additional N-glycosylation that occurred during processing made it impossible to estimate the changes in molecular weight due

to processing. It has been possible to demonstrate COOH-terminal processing of PLAP in a cell-free system (3, 4). However, even in such a system the large size of PLAP and the extent of glycosylation do not permit estimation of changes in molecular weight resulting from the sequential loss of NH₂- and COOH-terminal peptides and addition of the PI-G moiety during processing. To overcome this problem, we produced an engineered protein derived by deletion of about 60% of the coding sequences of PLAP cDNA (5). The modified cDNA codes for a pro form of the protein containing 208 residues (prominiPLAP); this compares to 513 residues for wild-type proPLAP. PreprominiPLAP (Fig. 1) contains each of the epitopes used to monitor processed products immunologically, as well as the NH₂- and COOH-terminal signal peptide sequences present in wild-type PLAP. COOH-terminal processing of preprominiPLAP in cell-free systems is specific, as was shown by the appearance of a single band possessing the immunological characteristics of mature PLAP, as well as the requirement for a small amino acid at the cleavage/attachment site. The latter is evident from inspection of other characterized PI-G-tailed proteins (6) and from site-directed mutational studies (4, 5).

In this report the processed products of preprominiPLAP and of several COOH-terminal truncated forms of this protein have been characterized. In addition, evidence is presented that COOH-terminal cleavage of full-length preprominiPLAP *in vitro* is accompanied by incorporation of the PI-G moiety.

MATERIALS AND METHODS

Construction of pGEM-4Z/MiniPLAP Truncation Mutants. A series of deletion mutants of wild-type PLAP 513 were used as starting material. Details of construction of pBC12/PLAP COOH-terminal deletions at residues 507, 502, and 489 were described earlier (2). Details of pGEM-4Z/miniPLAP 208 construction from wild-type pGEM-4Z/PLAP have also been described (5). In the present series, a 0.74-kilobase *Hind*III/*Stu* I fragment derived from pGEM-4Z/Δ489 was ligated into the 3.0-kilobase *Hind*III/*Stu* I fragment of pGEM-4Z/miniPLAP 208. The resulting plasmid is pGEM-4Z/Δ184. For pGEM-4Z/Δ202 and Δ197 miniPLAP, a 0.69-kilobase *Stu* I/*Asp*718 (used instead of *Kpn* I) fragment was purified from the pBC12 B1/PLAP 507 and 502 mutants. This fragment was ligated into the *Stu* I/*Asp*718 site of pGEM-4Z/miniPLAP 208. The resulting recombinant plas-

Abbreviations: PLAP, placental alkaline phosphatase; PI-G, phosphatidylinositol-glycan; RM, rough microsomal membranes; PI-PLC, inositol-specific phospholipase C; PI-PLD, inositol-specific phospholipase D.

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FIG. 1. Schematic presentation of preminiPLAP and COOH-terminal truncated mutants. Asp-179, the site of PI-G attachment deduced from studies in intact cells, is shown as a square, and the signal peptide, which is cleaved in the process, is shown as white on black. The arrows represent the sites of cleavage of the NH₂- and COOH-terminal signal peptides. Peptide sequences that were used to generate site-specific antibodies are overlined; a star designates the site of coupling of the synthetic peptide to the carrier protein. The antibodies (NH₂Ab, EndoAb, and ExoAb, antibodies directed to the NH₂-terminal, endo, and exo epitopes, respectively) react with the products of translation and processing in the following manner: preminiPLAP is NH₂Ab negative, EndoAb positive, and ExoAb positive; prominiPLAP is NH₂Ab positive, EndoAb positive, and ExoAb positive; mature miniPLAP is NH₂Ab positive, EndoAb positive, and ExoAb negative.

mids are pGEM-4Z/Δ202 and Δ179 miniPLAP. The following procedure was used to prepare pGEM-4Z/Δ179 miniPLAP cDNA. The plasmid pGEM-4Z/miniPLAP 208 was treated with *Bgl* II and *Hind*III to form two fragments, 2984 base pairs (bp) and 1036 bp. Both fragments were gel purified, electroeluted, and concentrated using Elutipis (Schleicher & Schuell). The 1036-bp fragment contained the codon for amino acid 180, which was to be converted to a stop codon. This was accomplished by site-directed mutagenesis employing PCR with the overlap extension technique described by Ho *et al.* (7) modified to use Vent polymerase (New England Biolabs). A pair of synthetic oligonucleotides containing a stop codon at position 180 were used. After the initial PCR reaction, the two subfragments, 737 bp and 323 bp, were gel purified and eluted with the aid of Spin-X tubes (Costar). DNA concentrations were estimated by ultraviolet absorption, and the two were mixed mole for mole in the final PCR reaction. The resultant 1036-bp fragment was gel purified, eluted from the gel in Spin-X tubes, ethanol precipitated, and treated with *Bgl* II and *Hind*III to regenerate the proper ends for ligation with the original 2984-bp fragment. The Δ179 miniPLAP cDNA was then used to transform competent DH5α cells (New England Biolabs), and mutant colonies were identified by restriction analysis and sequencing (8).

Biological Preparations. The miniPLAP plasmids were linearized with *Hind*III and transcribed using a Riboprobe II (Promega) kit. The mRNA was purified from the transcription reaction mixture by using an RNAid kit (Bio 101, La Jolla, CA). Rough microsomal membranes (RM) were prepared from Chinese hamster ovary cells as described (5) with the following modification. Cells were grown in suspension culture to a density of 10⁶ cells per ml. Six-liter batches were harvested in each run.

Translation and Processing of Nascent Forms of MiniPLAP. Cell-free translation reactions were carried out by using rabbit reticulocyte lysate as described by Pelham and Jackson (9) with modifications (3, 5). Typically, a 25.0-μl reaction mixture contained 12.5 μl of nuclease-treated lysate, 0.75 μl of a mixture of amino acids except methionine (each at 1 mM), 0.5 μl of RNase inhibitor at 40 units/ml (from Promega), 1.75 μl of [³⁵S]methionine (15 mCi/ml, 1100 Ci/mmol; 1 Ci = 37 GBq; Amersham), and 100 ng of mRNA. Two and one-half microliters of a 10× translation buffer (200

mM potassium acetate, 8 mM magnesium acetate, and protease inhibitors) was then added to this mixture. The mixture of protease inhibitors routinely contained aprotinin, antipain, bestatin, chymostatin, leupeptin, and pepstatin, each at 2 μg/ml.

For processing, RM [stored frozen at -70°C in a buffer containing 250 mM sucrose, 50 mM triethanolamine (pH 7.5), and 2 mM dithiothreitol] were thawed quickly, and 4–5 μl of the suspension was added to the translation reaction. Absorbance at 280 nm in 1% (wt/vol) SDS was used as an index of membrane concentration. The final concentration of membranes in each reaction mixture was usually maintained at about 8–10 absorbance units per ml. The reaction mixture was preincubated at 30°C for 2 min before adding the membranes. Typically, all reactions were incubated for 90 min at 30°C. Immunoprecipitations of translated samples with various antisera were carried out essentially as described (3, 5).

Electrophoresis and Fluorography. SDS/PAGE was performed according to Laemmli (10) with conditions as described (5). Quantitative densitometry was performed using an LKB laser densitometer.

Phospholipases. Inositol-specific phospholipase C (PI-PLC) was purified from *Bacillus thuringiensis* (11). Inositol-specific phospholipase D (PI-PLD), purified from bovine serum by the method of Huang *et al.* (12), was supplied by K. S. Huang.

Phase Distribution. Translation was carried out in the presence of miniPLAP 208 or Δ179 mRNA, scaling up the volume of the reaction to 100 μl. After 90 min of incubation at 30°C, the reaction mixture was chilled on ice, layered over 0.3 ml of a cushion [0.5 M sucrose, 50 mM triethanolamine (pH 7.5), 80 mM potassium acetate, 1 mM dithiothreitol, and a mixture of protease inhibitors each at 1 μg/ml], and centrifuged for 15 min at 80,000 rpm in a Beckman TL-100 ultracentrifuge using a TLA 100.2 rotor. The recovered RM were resuspended in 200 μl of TBS [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl] containing 1% Triton X-114, precondensed as described by Bordier (13), and sonicated 3 times for 10 sec at 35% intensity. Aliquots of RM suspension were incubated in the presence or absence of PI-PLC or PI-PLD for 3 h at 37°C with constant mixing. For PI-PLC cleavage, 5 units of the enzyme was used; the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, protease

FIG. 2. Translation and processing of preminiPLAP mRNA 208 and the truncated mutants. After incubation, aliquots were taken for immunoprecipitation with either a polyclonal antibody (Poly Ab) or an antibody directed to the NH₂-terminal epitope (NH₂ Ab). The polyclonal antibody reacts with all forms of miniPLAP and the mutants; the antibody directed to the NH₂-terminal epitope does not react with the prepro forms. $M_r \times 10^{-3}$ are indicated at right.

inhibitors at 1 $\mu\text{g}/\text{ml}$, and 0.1% Triton X-114. For PI-PLD cleavage, 60 ng of enzyme was added, and the reaction mixture contained 40 mM Tris maleate (pH 7.0), protease inhibitors at 1 $\mu\text{g}/\text{ml}$, and 0.1% Triton X-114. After incubation, Triton X-114 was added to 200 μl of a reaction mixture to a final concentration of 1%, and phase separation was performed as described by Bordier (13). The aqueous and detergent phases were then immunoprecipitated and analyzed by SDS/PAGE and fluorography.

RESULTS AND DISCUSSION

Fig. 1 shows the COOH termini of preminiPLAP and several truncated mutants. The mRNAs corresponding to preminiPLAP and the mutants were incubated with the cell-free processing system. As shown in Fig. 2, all the products of processing were clearly resolved by SDS/PAGE, even those that differed by only five or six amino acids. As can be seen in Fig. 2 and Table 1, preminiPLAP 208 and the $\Delta 202$ truncated mutant yielded three products. From their immunoreactivities and migration on gels, these were identified as the prepro form, the pro form, and mature miniPLAP. However, each of the shorter truncated mutant mRNAs yielded only two protein products. By analogy these are called the prepro form and the pro form. As would be expected from the sites of truncation (Fig. 1), neither of the two products reacted with antibody directed to the exo epitope (data not shown). The individual nascent proteins (prepro forms) were identified by their inability to react with antibody directed to the NH₂-terminal epitope (Fig. 2). On the other hand, the corresponding processed products had lost the NH₂-terminal signal peptide, which made the NH₂ epitope available for reaction with NH₂ antibody. It is apparent that the three smallest truncated mutants ($\Delta 197$, $\Delta 184$, and $\Delta 179$) were processed only at their NH₂ termini and did not undergo further cleavage at their COOH termini.

Of great interest is the fact that the same mature protein was derived from processing of both mRNA 208 and $\Delta 202$ and

that this product migrated with a higher molecular weight than the final products derived from mRNAs $\Delta 184$ and $\Delta 179$. From this we can conclude that processing of miniPLAPs 208 and $\Delta 202$ in the cell-free system is not simply due to cleavage of the COOH-terminal signal peptide at residue 179. If that were so, the mature product should be identical to that produced by the $\Delta 179$ mutant. Cleavage of prominiPLAP at residue 179 and addition of a PI-G moiety would be expected to yield a product whose M_r is larger than that of the processed form of $\Delta 179$ by about 1200–1500. The observed M_r values were 23,200 for $\Delta 179$ and 24,700 for mature miniPLAP.

There is additional evidence that the mature product derived from processing of preminiPLAP 208, and presumably $\Delta 202$, contains the PI-G moiety. When subjected to phase distribution between Triton X-114 and water, the mature protein resulting from translation and processing of miniPLAP 208 mRNA was found to be distributed equally between the detergent phase and water (Fig. 3). After treatment with either phospholipase C or D, which specifically cleaves the lipid moiety from PI-G-tailed proteins, most of the

FIG. 3. Phase distribution. After incubation, aliquots were allowed to distribute between the Triton X-114 phase and the aqueous phase prior to and after treatment with PI-PLC or PI-PLD. Each phase was then immunoprecipitated with polyclonal antibody, and the precipitates were subjected to SDS/PAGE. The bar graphs represent densitometric measurements obtained by scanning the gels. The mature form was measured in the case of 208, and the pro form was measured in the case of $\Delta 179$. The latter is equivalent to mature miniPLAP minus the PI-G moiety. Solid bars represent the aqueous phase; hatched bars represent the organic phase.

Table 1. Observed molecular weights of the products of processing of preminiPLAP mRNA and truncated mutants

Product	mRNA used for translation				
	208	$\Delta 202$	$\Delta 197$	$\Delta 184$	$\Delta 179$
Prepro	28,000	27,800	26,900	25,100	24,600
Pro	26,900	26,600	26,000	24,300	23,200
Mature	24,700	24,700	—	—	—

The M_r values are derived from the data in Fig. 2. It should be noted that these values are higher by ≈ 3000 than the molecular weights based on amino acid composition. This discrepancy is most likely due to inherent differences in gel migration between the miniPLAP proteins and the protein standards that were used for comparison.

mature protein appeared in the aqueous phase. PI-G-tailed proteins are characteristically hydrophobic and become hydrophilic on treatment with inositol-specific phospholipases (14). By contrast, the product resulting from mutant $\Delta 179$, which contains no COOH-terminal signal peptide, appeared mainly in the aqueous phase, and its distribution was not influenced by either phospholipase. The prepro and pro forms of miniPLAP 208 (Fig. 3 *Upper*), which should have contained no PI-G moiety, were also unaffected by the phospholipases.

The present experiments with miniPLAP and mutants of miniPLAP in a cell-free system confirm earlier experiments with wild-type PLAP in intact cells by showing that the size of a COOH-terminal hydrophobic peptide is critical for it to be processed to a PI-G-tailed form. Below a certain minimal size (in the case of miniPLAP, ≈ 20 residues), a hydrophobic COOH-terminal sequence no longer serves as a signal for PI-G tailing or cleavage by COOH-terminal "transamidase." Thus the truncation mutants $\Delta 197$, $\Delta 184$, and $\Delta 179$ do not contain a functional COOH-terminal signal peptide and are processed only at their NH_2 termini as typical secreted proteins. In the case of the PI-G-anchored protein, decay accelerating factor, it was shown that significant truncation of the COOH-terminal signal peptide abolishes membrane anchoring in intact cells (15).

The pro forms derived from preprominiPLAPs 208 and $\Delta 202$ are both cleaved at residue 179, yielding the same mature product. Since the latter is larger than the product derived from the truncated mutant preprominiPLAP $\Delta 179$, it must contain an additional substituent. The distributions of the various forms of 208 and $\Delta 179$ between Triton X-114 and water before and after treatment with PI-PLC and PI-PLD are fairly good evidence that PI-G tailing accompanies cleavage when the size of the COOH-terminal peptide is of a sufficient length to act as a signal for COOH-terminal processing. The most direct evidence for incorporation of PI-G during cleavage of prominiPLAP comes from experiments with RM prepared from mutant T-cell hybridomas that do not produce intact PI-G (16). We have recently shown that in such cells exogenously added PI-G is an obligatory cosubstrate for COOH-terminal processing and is actually incorporated into the mature product (K.K., R.A., D.C., E. T. H. Yeh, L. Brink, and S.U., unpublished results). We are now convinced that the mature products of PLAP and miniPLAP that were produced by cell-free processing in earlier reports (3–5)

were also PI-G tailed. In fact, Mayor *et al.* (17) have recently demonstrated that a ^3H -labeled PI-G precursor could be transferred *in vitro* onto trypanosomal variant surface glycoprotein.

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