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Selectivity at the cleavage/attachment site of phosphatidylinositol-glycan anchored membrane proteins is enzymatically determined

(alkaline phosphatase/site-directed mutagenesis/transamidase/COOH-terminal processing)

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ABSTRACT **Nascent precursors of phosphatidylinositol-glycan (PI-G)-linked membrane proteins contain a hydrophobic COOH-terminal sequence of 15–30 residues that is eliminated during processing to yield a newly exposed COOH terminus to which the PI-G moiety is added. There is no consensus as to the primary structure of the terminal peptide but there is a specific requirement for the amino acid destined to become the COOH terminus. In nascent human placental alkaline phosphatase (PLAP), the PI-G tail is attached to Asp-484. Site-directed mutants with glycine, alanine, cysteine, serine, or asparagine (category I) at residue 484 become PI-G tailed, appear in the plasma membrane, and are enzymatically active when expressed in COS cells. Although mutants with glutamic acid, glutamine, proline, tryptophan, leucine, valine, phenylalanine, threonine, methionine, and tyrosine (category II) are expressed equally well, only small amounts appear on the plasma membrane. Furthermore, they are not PI-G tailed and have little alkaline phosphatase activity. Studies with truncated PLAP-489 rule out nonspecific conformational changes in category II mutant proteins as a reason for their failure to be processed in COS cells and point to a specific COOH-terminal processing enzyme. Direct evidence that the selectivity for category I amino acids is enzymatically determined was obtained in a cell-free translation/processing system by using rabbit reticulocyte lysate and CHO cell rough microsomal membranes. In this *in vitro* system, both category I and category II mutants of PLAP-513 were translated, glycosylated, and cleaved by NH_2-terminal signal peptidase. However, an additional and selective cleavage at residue 484 was observed only with category I mutants.**

Although phosphatidylinositol-glycan (PI-G)-linked membrane proteins were only discovered within the last few years, ≈40 such proteins are now known (1–3). From the few PI-G-linked proteins that have been fully characterized, it is apparent that, in each case, the nascent precursor contains a COOH-terminal sequence that is largely hydrophobic and is eliminated during processing to yield a new COOH terminus. The PI-G moiety becomes attached through an ethanolamine residue to the amino acid at the newly exposed COOH terminus. It has been generally considered that a transferase or transamidase type of enzyme is responsible for peptide bond cleavage and simultaneous addition of the PI-G tail (1, 4, 5). From structural data of characterized PI-G-tailed proteins and from studies with mutant cDNAs transfected into mammalian cells (6), it is now apparent that for PI-G tailing and membrane insertion to occur the nascent forms must contain a hydrophobic COOH terminus of a certain minimum size (15–30 amino acids). However, there is no consensus as to the primary structure of the COOH-terminal signal peptides.

There is apparently a more specific requirement for the internal amino acid in the nascent protein that is destined to become the COOH terminus after cleavage and to accept the PI-G moiety. Thus far, only glycine, alanine, serine, cysteine, aspartic acid, and asparagine have been reported as acceptors for the PI-G moiety (3). In human placental alkaline phosphatase (PLAP), the PI-G tail is attached to an aspartic acid, which represents Asp-484 of the nascent protein (7, 8). In a previous study (9), we found that site-directed mutants with glycine, alanine, serine, cysteine, or asparagine (category I) at residue 484 of PLAP became PI-G tailed, appeared in the plasma membrane, and were enzymatically active (see Fig. 1). Mutant forms of PLAP with other amino acids substituted at residue 484, including glutamic acid, glutamine, proline, tryptophan, leucine, valine, phenylalanine, threonine, methionine, and tyrosine (category II), were expressed as well as wild-type PLAP-513, but only traces appeared on the plasma membrane in PI-G-tailed form. In addition, category II mutants exhibited little or no alkaline phosphatase activity. The present studies were carried out to determine the reason for the high degree of selectivity at the PI-G-tailing site observed in transfected COS cells and to determine whether the same specificity is observed in a cell-free translation and processing system (4).

MATERIALS AND METHODS

Recombinant Plasmids and Mutagenesis. Asp-484 site-specific mutants of wild-type PLAP-513 were prepared as described (9). A secreted form of the deletion mutant PLAP-489 (see Fig. 1) was constructed and characterized by Berger *et al.* (6). Asp-484 site-specific mutants of PLAP-489 were prepared by oligonucleotide-directed mutagenesis of an M13-mp19/PLAP-489 vector by the method of Kunkel *et al.* (10), ensuring high efficiency of mutagenesis. The vector was constructed by subcloning a 1.6-kilobase (kb) *Bam*HI/*Kpn* I fragment of PLAP-489 cDNA into the corresponding restriction sites of the M13 multiple cloning site. Mutant clones were identified by DNA sequencing (11) and were plaque-purified. After M13 *in vitro* mutagenesis, a 1.6-kb *Bam*HI/*Kpn* I PLAP-489 cDNA fragment was recloned in the mammalian expression vector pBC12BI (6).

Cell Culture and Transient Expression of PLAP Proteins. COS 7 cells were maintained in culture and transfected with various pBC12BI/PLAP plasmids in 36-mm wells by the DEAE-dextran method (12). Approximately 72 hr after transfection, the cell medium was removed and centrifuged for 3 min at 14,000 × *g* to remove cell debris. The cells were washed and homogenized as described (13). Media and cell homogenates were then assayed for PLAP activity with *p*-nitrophenyl phosphate used as a substrate (14) and 10 mM

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Abbreviations: PLAP, placental alkaline phosphatase; PI-G phosphatidylinositol-glycan; RM, rough microsomal membranes.

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L-homoarginine to inhibit endogenous, nonplacental alkaline phosphatases normally present in COS cells (13).

Metabolic Labeling of Transfected COS Cells and Immunoprecipitation of PLAP Proteins. Steady-state metabolic labeling with [^{35}S]methionine (Amersham) of transfected COS cells was performed ≈60 hr posttransfection. Cells were preincubated for 1 hr in methionine-free Dulbecco's minimal essential medium supplemented with 5% dialyzed fetal calf serum (GIBCO). Fresh medium, containing [^{35}S]methionine (1440 Ci/mmol; 100 μCi per 35-mm dish; 1 Ci = 37 GBq), was then added and incubation was continued for another 12 hr. The culture medium was then removed and cleared by centrifugation; the cells were lysed with 2% $NaDodSO_4$/5% 2-mercaptoethanol (vol/vol). Immunoprecipitation of labeled PLAP proteins and analyses by $NaDodSO_4$/PAGE followed by fluorography were performed as described (15). Proteins were immunoprecipitated with a 1:1000 dilution of a purified polyclonal antibody to mature PLAP (Accurate Chemicals) or a 1:500 dilution of an antiserum containing site-directed polyclonal antibodies to the exo epitope. The latter recognizes the nonapeptide at the COOH terminus (residues 505–513) of the 29-residue COOH terminal signal peptide (see Fig. 1).

Preparation of PLAP mRNAs. For expression in the cell-free system and subsequent processing *in vitro*, PLAP-513 Asp-484 mutant cDNAs were subcloned in the transcriptional vector pGEM-4Z (Promega Biotec). A 2.2-kb *Eco*RI fragment containing mutagenized PLAP cDNA was cloned into the *Eco*RI site of pGEM-4Z, thus placing the PLAP gene under the control of SP6, a strong transcriptional promoter. The resulting plasmids were amplified and purified by CsCl equilibrium gradient centrifugation. PLAP mRNA was prepared with the Riboprobe-II (Promega Biotec) *in vitro* transcription system. Purified mRNAs were stored at −70°C in distilled water.

Preparation of Rough Microsomal Membranes (RM). Chinese hamster ovary (CHO) cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. RM preparations from confluent cultures were made as follows: cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were harvested either by scraping into PBS or detached with PBS containing 1 mM EDTA. Harvested cells were pelleted at 1000 × *g* and washed twice with PBS. Cells were resuspended in 10 mM Tris·HCl (pH 7.5) and incubated at 4°C for 5 min. The cell suspension was diluted with an equal vol of 600 mM sucrose/6 mM dithiothreitol and was homogenized with 10 strokes in a Dounce homogenizer. The suspension was centrifuged at 7700 × *g* for 10 min and the supernate was recentrifuged at 17,300 × *g* for 20 min. Rough microsomes were sedimented from the second supernate by centrifugation at 100,000 × *g* for 60–75 min. Essentially all the supernatant was removed by aspiration and the RM pellet was immediately stored at −70°C.

Translation and Processing of Nascent Forms of PLAP. Cell-free translation using rabbit reticulocyte lysate (Promega Biotec) was carried out as described by Pelham and Jackson (16) with minor modifications. Typically, a 25.0-μl translation mixture contained 12.5 μl of nuclease-treated rabbit reticulocyte lysate, 1.0 μl of 1 mM amino acid mixture minus methionine, 0.5 μl of RNase inhibitor at 40 units/μl (all supplied by Promega Biotec), 1.5–2.0 μl of [^{35}S]methionine (15 mCi/ml; 1100 Ci/mmol; Amersham), and 1–3 μg of PLAP mRNA. Five microliters of translation buffer (100 mM KCl/4 mM Mg^{2+}/50 mM sucrose/3 mM dithiothreitol/protease inhibitors) was then added. The cocktail of protease inhibitors routinely contained aprotinin, antipain, bestatin, chymostatin, leupeptin, and pepstatin (each at 2 μg/ml) and RNase inhibitor (5 units/ml). To emphasize the specific nature of COOH-terminal cleavage in the presence of RM, several other protease inhibitors were occasionally added to the basic cocktail (phenylmethylsulfonyl fluoride, L-1-tosylamido-2-phenylethyl chloromethyl ketone, and 7-amino-1-chloro-3-tosylamido-2-heptanone at 50 μg/ml).

For *in vitro* processing, the RM pellets were resuspended immediately before use by repetitive pipetting in the translation buffer and 5.0 μl of membrane suspension was added to the translation mixture. Absorbance at 280 nm in 1% (wt/vol) $NaDodSO_4$ was used as an index of membrane concentration. The final concentration of RM in reaction mixtures was usually 6.0–8.0 OD units. The reaction mixture was preincubated for 2 min at 30°C prior to the addition of either translation buffer or RM suspension. Incubations for translation and processing were performed at 30°C for 60–75 min, after which the samples were brought to 4°C. Immunoprecipitations of translated samples with the various antisera were performed essentially as described by Bailey *et al.* (4).

Electrophoresis and Fluorography of Products from the Cell-Free System. $NaDodSO_4$/PAGE was performed as described by Laemmli (17) with minor modifications. Gels

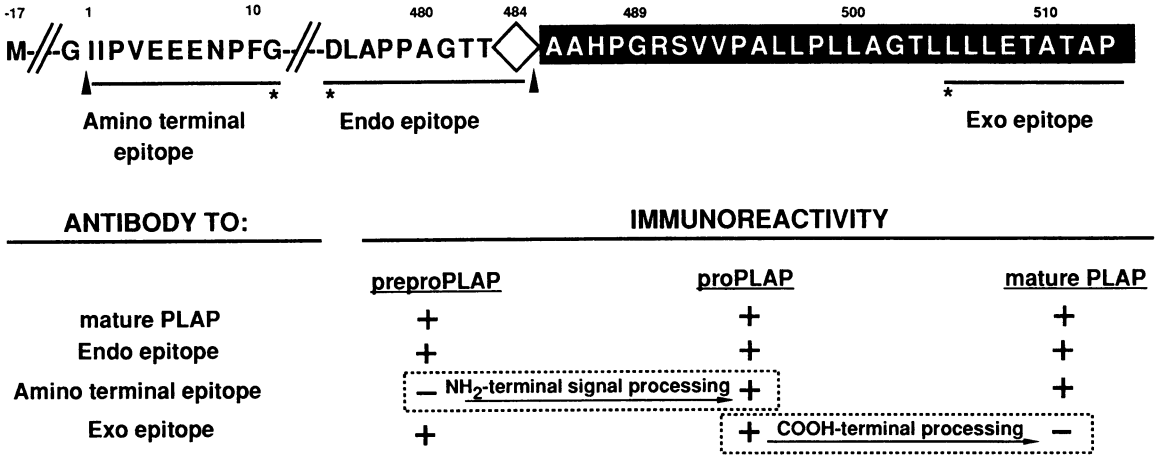

FIG. 1. Schematic representation of prepro-PLAP-513 with the COOH-terminal signal peptide, which is removed during processing, shown as white on black. ◇, Residue 484, the site of PI-G tailing as well as the site of mutagenesis in these studies. Underlined sequences represent the peptides used to develop the site-specific antibodies. Sites of conjugation of the synthetic peptides to the carrier protein used for immunization are indicated by stars. Category I or category II amino acids were substituted at residue 484 in the site-directed mutants. The deletion mutant PLAP-489 terminated at residue 489 of wild-type PLAP. Arrowheads indicate sites of cleavage of the NH_2-terminal and COOH-terminal signal peptides.

(7.5% or 10.0%) were cast using Bio-Rad PROTEAN II xi slab gel systems. Resolving gels were 0.75 mm thick and 12.0 cm long with 1.5 cm of stacking gel. They were run at 8°C and at constant current conditions: 14–15 mA during stacking and 18 mA for resolving. Gels were fixed, treated with Enhance II (New England Nuclear), dried, and exposed to XAR-2 film (Eastman Kodak) at −70°C.

RESULTS

The observed selectivity of the PI-G-tailing system for category I type amino acids at the cleavage/attachment site of PLAP led us to postulate two possible scenarios (9). One possibility was that to be processed correctly the protein, in this case PLAP, must be in its "native" conformational state. To maintain this stage, only small amino acids, as in category I, could be tolerated at residue 484 (Fig. 1). Substitution of large amino acids at that site would lead to improper folding so that the precursor protein would no longer be enzymatically active or capable of being processed to a PI-G-tailed form. In other words, could a single amino acid substitution far removed from the catalytic site (residues 91–93), and late in translation, by itself lead to a major conformational change that would alter both the enzymatic activity of PLAP as well as its ability to be recognized by the putative "PI-G transamidase"? One way to answer this question was to utilize a COOH-terminal truncated form of PLAP. We had found earlier that when the corresponding truncated cDNAs were expressed in COS cells, the truncated PLAPs were no longer PI-G tailed but were secreted into the medium in an enzymatically active form (6). We utilized one of these truncated mutants, PLAP-489, into which category I and category II type amino acids were substituted at residue 484 (see Fig. 1). When the corresponding mutant cDNAs were expressed in COS cells all the site-directed mutants of PLAP-489 (category I and category II) were secreted into the medium in comparable amounts (Fig. 2). The smaller amounts of PLAP-489 in cells represent protein in the process of synthesis and translocation through the secretory pathway. Furthermore, as shown in Table 1, all site-directed, secreted mutants of PLAP-489 exhibited high alkaline phosphatase activity. By contrast only category I site-directed mutants of full-length PLAP-513 demonstrated high alkaline phosphatase activity. Thus, the presence of large amino acids at residue 484 of nascent PLAP does not, of itself, lead to improper folding, as determined by both phosphatase activity of PLAP-489 mutant proteins as well as by their ability to be secreted.

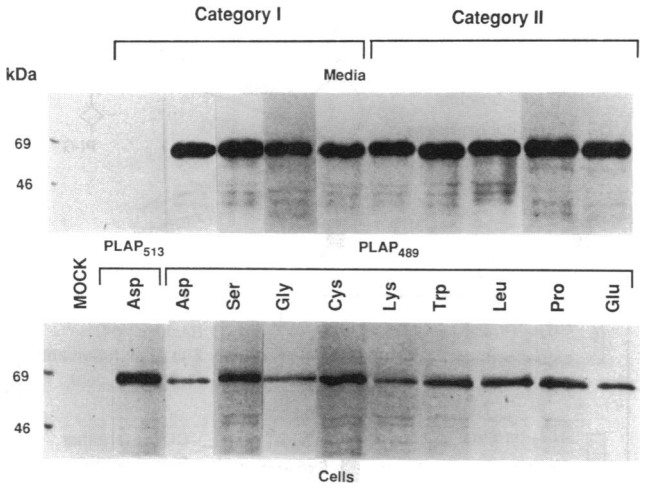

FIG. 2. Expression of truncation mutant PLAP-489 and selected site-directed mutants (residue 484) by COS cells. ^{35}S-labeled proteins were precipitated from cell lysates and/or culture media by immunoprecipitation with polyclonal anti-PLAP antisera and subjected to $NaDodSO_4$/PAGE. Expression of wild-type PLAP-513 is shown for comparison.

Table 1. Relative values of alkaline phosphatase activity of both the membrane-bound (PLAP-513) and secreted (PLAP-489) forms and of the corresponding Asp-484 site-directed mutants

Mutant	Alkaline phosphatase activity, relative values	
	Membrane-bound PLAP-513	Secreted PLAP-489
Category I		
Asp	1	1
Ser	2.59 ± 0.34	1.15 ± 0.28
Gly	1.17 ± 0.16	0.74 ± 0.16
Cys	0.39 ± 0.16	0.60 ± 0.01
Category II		
Lys	0.04 ± 0.02	0.77 ± 0.20
Trp	0.04 ± 0.01	1.03 ± 0.01
Leu	0.13 ± 0.01	1.26 ± 0.04
Pro	0	1.47 ± 0.32
Glu	0.05 ± 0.02	0.72 ± 0.06

Values for the aspartic acid forms are arbitrarily taken as 1.0. The enzyme activity of the membrane-bound PLAP-513 aspartic acid variant was 1.45 milliunits per mg of protein. For the secreted PLAP-489 aspartic acid variant, the observed enzyme activity was 82 milliunits per ml of medium.

An alternative explanation of the selectivity of the cleavage/attachment site of PLAP that was observed in the mammalian expression system is that it results from the substrate specificity of the putative transamidase. It should be noted that NH_2-terminal signal peptidase is relatively specific, recognizing only glycine, alanine, and serine residues (18, 19). To test for specificity with respect to COOH-terminal processing an *in vitro* translation and processing system had been developed in our laboratory (4). mRNA, produced from the corresponding cDNA, is translated by rabbit reticulocyte lysate in the presence of CHO cell microsomes. By utilizing appropriate site-directed antibodies (as shown in Fig. 1), it was possible to demonstrate cleavage of wild-type PLAP-513 at Asp-484 and loss of the 29-residue COOH terminus of the nascent protein. As shown in Fig. 3, three PLAP-related products appeared after incubation with microsomes. The one with the smallest molecular mass represents unprocessed prepro-PLAP, which, from its immunological reactivity, contains both the NH_2- and COOH-terminal signal peptides. The pair of slower-running protein bands are glycosylated forms of PLAP. The upper band is pro-PLAP, which has lost its NH_2-terminal signal peptide but retains the COOH terminus. The lower band represents mature PLAP that no longer contains either NH_2- or COOH-

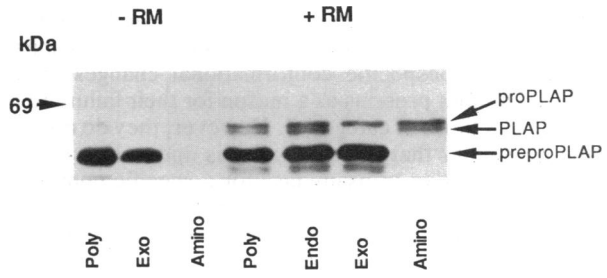

FIG. 3. Processing of wild-type prepro-PLAP-513 (produced in rabbit reticulocyte lysate) by CHO cell RM in the presence of protease inhibitors. After incubation in the presence and absence of RM, aliquots were taken for immunoprecipitation with the specific antibodies (poly, antibody to mature PLAP; exo, exo-Ab; endo, endo-Ab; amino, antibody to NH_2 terminus of mature PLAP). Immunoprecipitates were subjected to $NaDodSO_4$/PAGE and fluorography.

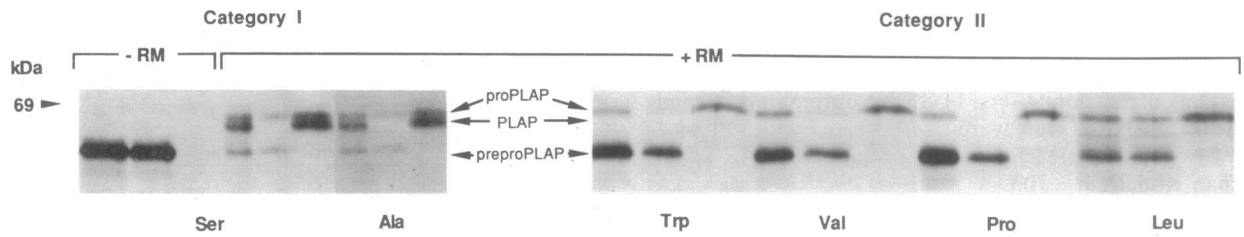

FIG. 4. Processing of residue 484 mutant forms of prepro-PLAP in the presence of CHO cell RM. The appropriate mRNA was added to the complete system and, after incubation, aliquots were taken from each mutant sample for precipitation with the different antibodies. This was followed by $NaDodSO_4$/PAGE and fluorography. The first three bands are the results using PLAP-513/Ser-484 mRNA in the absence of RM; the second three bands are in the presence of RM. Each subsequent set of three bands represents (from left to right) immunoprecipitates with polyclonal, exo, and amino antibodies.

terminal signal peptides. The latter still reacts with endo-Ab (antibody to residues 475–484 of prepro-PLAP), indicating that cleavage is after residue 484. The results obtained with selected category I and category II mutants of PLAP-513 are shown in Fig. 4. Category I mutants containing serine and alanine at residue 484 (as well as glycine and cysteine; data not shown) yielded two processed products, the pro- and mature forms. In contrast, each of the category II mutants, tryptophan, valine, proline, and leucine, yielded only one processed product that was N-glycosylated and cleaved by NH_2-terminal signal peptidase (pro-PLAP). They did not yield the mature form of PLAP, as would be indicated by loss of the exo epitope and a corresponding decrease in size.

DISCUSSION

The two different studies reported here provide convincing evidence that the selectivity for category I amino acids at the cleavage/attachment site (residue 484) of nascent PLAP, originally observed in intact cells (9), is due to specificity of the putative transamidase for small amino acids at the cleavage/attachment site and not to gross conformational changes in the nascent protein brought about by substitution with category II amino acids. The first series of experiments showed that mutant proteins that were not destined to be PI-G tailed (because they lacked an intact COOH-terminal signal peptide; PLAP-489) were secreted with full enzyme activity with either category I or category II substituents at residue 484. This is in contrast to earlier studies with mutant forms of full-length PLAP-513 (which do contain an intact COOH-terminal signal peptide), in which category II substituents at residue 484 gave rise to proteins that were not located at the cell surface, were not PI-G tailed, and were essentially devoid of alkaline phosphatase activity. An obvious conclusion from the present experiments is that substitution of a category II amino acid at residue 484 during translation does not influence the conformation of the protein, at least with respect to enzyme activity and secretion.

The findings from expression studies in intact cells apparently rule out nonspecific conformational changes in the category II mutant proteins as a reason for their failure to be processed like those of category I. However, they do not rule out the possibility that the selectivity is due to the requirement by the putative transamidase for a specific conformation localized to the cleavage/attachment site. The most direct evidence of substrate specificity of an enzyme is to study pure substrates and pure enzymes. However, at this stage it is not possible to do so with PI-G transamidase. The simplest cell-free system we have used to demonstrate the enzyme activity generates the substrate prepro-PLAP from PLAP mRNA in a rabbit reticulocyte lysate translation system. A microsomal fraction from CHO cells contains the enzyme and other possible factors that are required for processing. With this system we have not as yet demonstrated the incorporation of the PI-G moiety. However, we have demonstrated specific cleavage of nascent PLAP at residue 484 that is not influenced by peptidase inhibitors (4). Evidence that this cleavage represents PI-G transamidase activity was obtained with site-specific antibodies. Thus, after microsomal action only one mature product appeared (Fig. 3) that had lost the exo epitope, indicating loss of the 29-residue COOH-terminal signal peptide. However, the product fully retained reactivity with the endo Ab, the epitope that ends with Asp-484 (Fig. 1) (4). The band corresponding to mature PLAP (Fig. 3) had also lost its NH_2-terminal signal peptide as shown by an ability to react with NH_2-terminal antibody. That epitope is inaccessible to the antibody when present as an internal sequence in prepro-PLAP. In the present studies, Asp-484 site-directed mutants of prepro-PLAP were generated in the presence of CHO microsomes. The fact that only the aspartic acid, serine, and alanine mutants (category I) were cleaved at the specific site and the proline, valine, leucine, and tryptophan mutants (category II) were not provides the most direct evidence that the selectivity for PI-G tailing observed in whole cells has its basis in the specificity of an enzyme, most probably a transamidase. Fig. 5 is a schematic presentation of the processing of prepro-PLAP and its residue-484 site-directed mutants. Whether or not PI-G is actually incorporated into mature PLAP in these studies has not yet been determined because the amounts formed per experiment (10–20 fmol) are

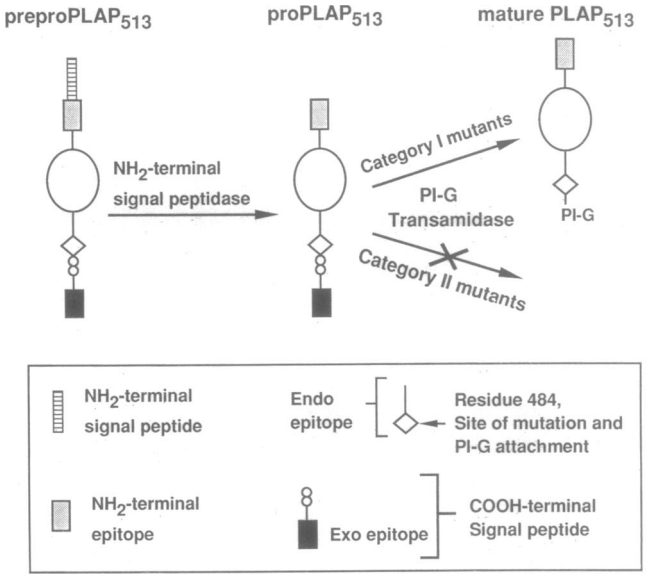

FIG. 5. Schematic presentation of the processing of prepro-PLAP-513 and its residue 484 site-directed mutants and the immunological rationale to characterize their products. 8 , Two category I amino acids (small amino acid domain) adjacent to the cleavage/attachment site.

too low to detect incorporation of the labeled components of PI-G (4).

We have recently engineered a shortened form of PLAP cDNA that encodes a protein devoid of glycosylation sites but with the same NH_2-terminal and COOH-terminal regions. The mRNA produced from this engineered form of PLAP cDNA should yield a nascent protein of 23.8 kDa. When translated in the presence of CHO RM and subjected to immunoprecipitation and PAGE, it yields three easily separable bands that correspond in both size and immunoreactivity to the prepro form, the pro form, and the mature form of the protein. In this system too, when substitutions were made at the site corresponding to residue 484 of PLAP, only category I mutants were processed to yield a mature form that had lost its COOH-terminal signal peptide, whereas category II mutants were not. Details of the production of the engineered form of PLAP and its application to studies on PI-G transamidase will be presented elsewhere (K.K., R.M., L.D.G., Mauricio Tamburrini, Larry Brink, and S.U., unpublished data).

Examination of the structures of the characterized PI-G-tailed proteins indicates two requirements for PI-G tailing. First, the nascent proteins are generally hydrophilic except for a hydrophobic COOH-terminal sequence, which may vary considerably in size and amino acid composition. In the case of PLAP mutants, if the hydrophobic sequence is followed by a hydrophilic sequence of significant size, the latter can act as a cytoplasmic domain and prevent cleavage and PI-G tailing (20). In such a mutant, the COOH-terminal hydrophobic sequence acts as a typical membrane-spanning region to anchor the protein. The second requirement, which comes from studies involving site-directed mutagenesis at residue 484 of PLAP as well as from observations of other PI-G-tailed proteins, is the presence of a small amino acid (category I) at the cleavage/attachment site. Thus far, there have been no exceptions for this category I amino acid requirement in fully characterized PI-G-tailed proteins (3). Other requirements for this type of processing may be the nature of the amino acid residues in the immediate vicinity of the site of the cleavage. In all PI-G-tailed proteins that have been fully characterized thus far, the small amino acid at the attachment site is followed by at least one, but generally two, other small amino acids (3, 21). In the case of PLAP, Asp-484, the cleavage site, is followed by Ala-485 and Ala-486 (see Fig. 1). The requirements for PI-G tailing are not rigid, merely a small amino acid domain (frequently three consecutive category I amino acids) followed by a hydrophobic chain of 15–30 residues. It is conceivable, therefore, that alternative PI-G-tailing sites (i.e., small amino acid domains) can be exposed and act as PI-G attachment sites by experimentally increasing or decreasing the length of the hydrophobic COOH-terminal signal peptide or by deleting the normal attachment site. The latter was apparently the case when a segment of the COOH-terminal signal peptide of decay-accelerating factor was excised (22). Mutational studies at sites other than residue 484, using both intact cells and cell-free processing, are needed to determine what additional requirements there may be at the COOH terminus for recognition by PI-G transamidase.

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