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Processing at the carboxyl terminus of nascent placental alkaline phosphatase in a cell-free system: Evidence for specific cleavage of a signal peptide

(membrane-anchored proteins/phosphatidylinositol glycan)

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ABSTRACT **Alkaline phosphatase is anchored to the plasma membrane by a carboxyl-terminal phosphatidylinositol glycan moiety. To investigate the biosynthesis of mature alkaline phosphatase, nascent human placental alkaline phosphatase was expressed in a cell-free system and used as substrate for *in vitro* processing by microsomal extracts. By monitoring the processed product with three site-directed antibodies, it was shown that microsomal extracts from CHO cells that contain other recognized processing activities also remove the carboxyl-terminal signal peptide from the preproenzyme in an apparently selective manner. This peptidase-like cleavage may be brought about by the action of a specific transamidase acting on the nascent protein in the absence of an appropriate phosphatidylinositol glycan cosubstrate.**

The alkaline phosphatases belong to a class of proteins that are anchored to the plasma membrane by a phosphatidylinositol glycan (PI-G) moiety (1, 2). In human placental alkaline phosphatase (PLAP), the PI-G moiety is attached to aspartic residue 484 of the 530-residue nascent protein (3, 21), indicating that a 29-residue peptide, the carboxyl-terminal signal peptide, is cleaved from the carboxyl terminus of the nascent protein during processing. Elimination of a carboxyl-terminal peptide apparently occurs during processing of other PI-G-tailed proteins (4, 5). To study the mechanisms involved in the addition of PI-G, we have expressed nascent PLAP in a cell-free system to serve as substrate for *in vitro* PI-G-tailing by microsomal extracts. Three site-specific antibodies that have been described (6) were used to monitor the various stages of processing (Fig. 1). Addition of microsomal extracts from CHO cells during translation in a eukaryotic system resulted in a product that was glycosylated and therefore larger than nascent PLAP, as shown by $NaDodSO_4$/PAGE. By monitoring the processed product with the various site-directed antibodies, it was shown that microsomal extracts had specifically removed both the amino- and carboxyl-terminal signal peptides from the preproenzyme (Fig. 1). The significance of this observed *in vitro* carboxyl-terminal processing is discussed from the standpoint of a proposed mechanism for PI-G-tailing.

MATERIALS AND METHODS

Materials. Routinely used reagents, enzymes, inhibitors, and detergents were obtained from commercial sources. Nuclease-treated rabbit reticulocyte lysate and the pGEM plasmid system were from Promega Biotec. Radiolabeled compounds were from Amersham and New England Nuclear.

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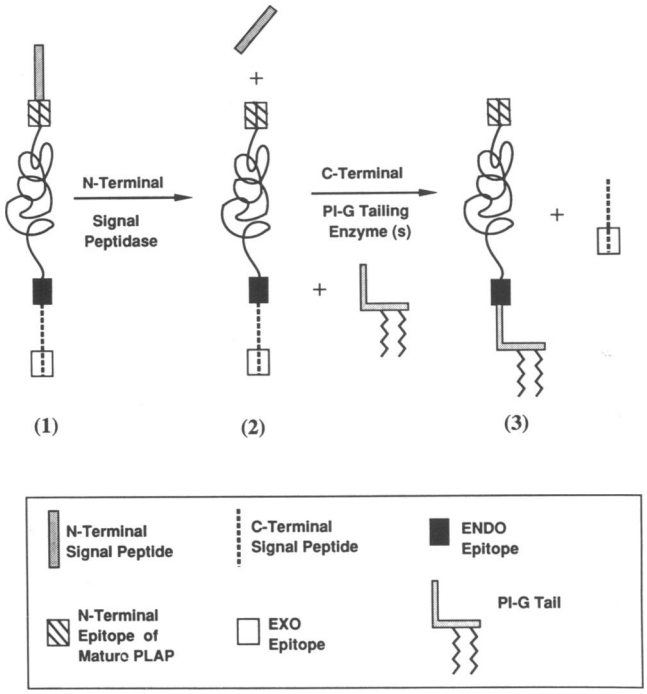

FIG. 1. Proposed model for processing of PLAP during translation. The 530-residue full-length preproprotein (1) contains all three site-specific epitopes: the blocked amino-terminal sequence and the endo and exo regions at the carboxyl terminus. Cleavage of the signal sequence at the amino terminus by microsomal signal peptidase results in proPLAP (2) that now readily reacts with antibody raised against the amino-terminal epitope of mature PLAP. Further microsomal processing at the carboxyl terminus cleaves a second peptide, the carboxyl-terminal signal peptide, which contains the exo-epitope, leaving the endo-epitope at the extreme carboxyl terminus of the mature protein. Concurrent with this cleavage (or shortly thereafter) the PI-G tail is added to complete the processing to mature PLAP (3).

Site-Directed Anti-Peptide Antibodies. Three site-specific antibodies were used (Fig. 2). The "exo-antibody" (exo-Ab) was generated against a synthetic peptide representing the last nine amino acids (residues 505–513) at the carboxyl terminus of the full-length nascent protein (preproPLAP). A second antibody, the "endo-antibody," was raised against a

Abbreviations: PI-G, phosphatidylinositol glycan; PLAP, placental alkaline phosphatase; exo-Ab, antibody to residues 505–513 at carboxyl terminus of preproPLAP; endo-Ab, antibody to residues 474–484 of preproPLAP; amino-Ab, antibody to amino terminus of mature PLAP.

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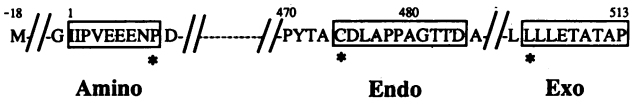


FIG. 2. Amino acid sequences of the three site-specific anti-peptide antibodies. Numbers above the sequence indicate the location of each residue in preproPLAP. Sites of conjugation of the peptides to carrier protein prior to immunization are indicated by an asterisk.

synthetic peptide representing residues 474–484 of prepro-PLAP. This epitope occurs upstream of the exo-epitope and terminates at the aspartate residue to which the PI-G tail is attached. The third antibody was raised against a synthetic peptide representing the first nine residues at the amino terminus of mature PLAP. The specificities and reactivities of these site-directed anti-peptide antibodies have been reported (6, 7). Affinity-purified rabbit polyclonal IgG raised against human PLAP (Accurate Chemicals, Westbury, NY) was used in comparative studies.

Production of Nascent PLAP. For the production of nascent PLAP in a eukaryotic system, a 2.3-kilobase (kb) cDNA insert containing the entire human PLAP coding region and 0.7 kb of 3′ untranslated region (8) was inserted into the pGEM plasmid (Promega Biotec). The plasmid was amplified and purified, and PLAP mRNA was prepared by using reagents and protocols provided with the pGEM vector; purified mRNA was stored at −70°C in distilled water. Cell-free translation using reticulocyte lysate was basically as described by Pelham and Jackson (9). A typical 50-μl assay mixture contained 17.5 μl of nuclease-treated rabbit reticulocyte lysate, 2 μl of unlabeled amino acids minus methionine (both as supplied by Promega Biotec), 1 μCi (37 kBq) of [^{35}S]methionine (800 Ci/mmol; Amersham) per ml, and 3–5 μg of RNA.

A second PLAP expression vector was constructed by using the prokaryotic expression plasmid pEV-vrf2 (10) into which the 2.3-kb PLAP cDNA was inserted. Nascent PLAP was then produced in a DNA-directed prokaryotic cell-free transcription/translation system according to directions provided by the supplier (Amersham).

Preparation of Microsomal Membranes. Chinese hamster ovary (CHO) cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Hazelton, Lenexa, KA). Microsomal membranes were prepared from confluent cultures as follows. After two washes of the cell layer with ice-cold phosphate-buffered saline (PBS), cells were harvested either by gentle scraping into PBS or detached with PBS containing 1 mM EDTA. Harvested cells were pelleted at 1000 × *g* and washed twice with PBS. The cell pellet was resuspended in 300 mM sucrose/3 mM dithiothreitol, and cells were homogenized with 10 strokes in a Dounce homogenizer. In some preparations, cells were swollen at 0°C for 5 min in 5 mM Tris (pH 7.5) prior to homogenization and then diluted with an equal volume of 600 mM sucrose/6 mM dithiothreitol. The suspension was centrifuged at 7700 × *g* for 10 min, and the supernate was recentrifuged at the same speed for 20 min. Microsomal membranes were sedimented from the second supernate by centrifugation at 100,000 × *g* for 60 min. The supernate was removed from the membranes as completely as possible by aspiration, and the membrane pellet was immediately stored at −70°C. CHO cells were used in all of the experiments reported. Membranes derived from WISH cells, JEG cells, canine pancreas, and rat liver were found to be less active or totally inactive with respect to processing at the carboxyl terminus.

Microsomal Processing. For use in *in vitro* processing, the membrane pellets were resuspended immediately before use by repetitive pipeting through a 200-μl micropipet tip in buffer composed of 100 mM KCl; 4 mM Mg^{2+}; 300 μM dithiothreitol; 10 mM Tris (pH7.5); bestatin, antipain, chymostatin, leupeptin, and pepstatin at 1 μg/ml; aprotinin at 2 μg/ml; and RNase inhibitor at 5 units/ml. Absorbance at 280 nm in 1% (wt/vol) $NaDodSO_4$ was used as a relative index of membrane concentration. In general, membranes were resuspended at concentrations such that A_{280} ranged from 10 to 50 units in the stock suspension, which was then diluted by one-fifth in the processing assay. Microsomal suspensions were added during or after initiation of translation as indicated in the various experiments. Translation and microsomal processing assays were usually performed at 30°C, after which the samples were brought to 0°C.

Characterization of Products Synthesized *in Vitro*. $NaDodSO_4$/PAGE was performed as described by Laemmli (11) with 7.5% gels. Gels were fixed, treated with fluor (Enlightning, New England Nuclear), dried, and fluorographed with XAR-2 film (Eastman Kodak) exposed at −70°C. Quantitative autoradiography was performed on a Zeineh soft laser-scanning densitometer (LKB).

Immunoprecipitation of translated samples was as follows. An equal volume of 0.1 M NaOH was added to the sample aliquot after translation, and this was further diluted with an equal volume of 8% $NaDodSO_4$/10 mM dithiothreitol. The diluted translation mix was then heated in a boiling water bath for 5 min, cooled to room temperature, and again diluted with an equal volume of water so that the sample contained 2% $NaDodSO_4$ and 2.5 mM dithiothreitol. This sample was diluted 1:9 with radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100/0.5% deoxycholate/0.2% $NaDodSO_4$/100 mM NaCl/1 mM EDTA/50 mM Tris·HCl, pH 7.5) and incubated with antibody overnight at 4°C. Protein A (IgGsorb, The Enzyme Center, Malden, MA) was then added to a final concentration of 1%, and the suspension was incubated for 30 min at room temperature. In some experiments, 2 μg of aprotinin per ml or 2 mM phenylmethylsulfonyl fluoride was included in the RIPA buffer and IgGsorb suspension. Bound immunocomplexes were eluted twice with $NaDodSO_4$/PAGE sample buffer for 5 min in a boiling water bath. The two elutions were pooled for $NaDodSO_4$/PAGE and fluorography. Control samples either contained no protease or 1% Triton X-100. For protease protection assays, samples were incubated for 30 min at 0°C with 10 μg of proteinase K per ml. Proteolysis was terminated by the addition of 1 mg of phenylmethylsulfonyl fluoride, and samples were analyzed by $NaDodSO_4$/PAGE and fluorography.

RESULTS AND DISCUSSION

PreproPLAP Is Synthesized by Rabbit Reticulocyte Lysate and Processed by CHO Microsomal Membranes. To characterize the products synthesized *in vitro* prior to use of the site-directed antibodies, PLAP mRNA was translated in rabbit reticulocyte lysates in the presence and absence of microsomes. Total translation samples were then analyzed by $NaDodSO_4$/PAGE (Fig. 3). In agreement with previous reports (12), we observed a radiolabeled product with a M_r ≈48,000 in the *in vitro* translations, which appeared as a doublet in the presence of microsomal membranes (Fig. 3, lanes 1 and 3). Since this product was not RNA dependent, it was not considered further in our characterization studies. A single major labeled product corresponding to preproPLAP was formed in lysates containing PLAP mRNA only (Fig. 3, lane 2). A larger processed form of PLAP was produced in the presence of RNA and microsomes (Fig. 3, lane 4 vs. lane 2). The estimated molecular weight of preproPLAP based on sequencing data (530 residues) is 57,500. The unprocessed protein synthesized *in vitro* exhibited a M_r of 59,000 as determined by $NaDodSO_4$/PAGE. Processing by microsomes

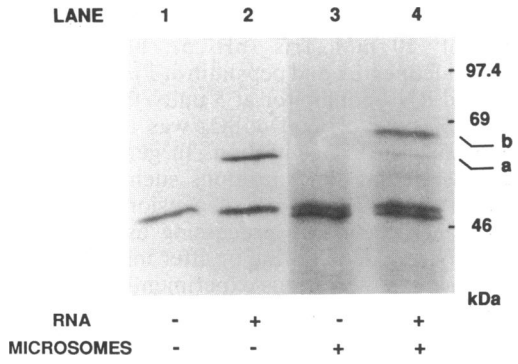


FIG. 3. *In vitro* translation of PLAP mRNA in rabbit reticulocyte lysates in the presence (lanes 3 and 4) and absence (lanes 1 and 2) of CHO microsomal membranes (final concentration, 5 units/ml). Incubation was for 90 min at 30°C followed by immunoprecipitation with polyclonal antibody and $NaDodSO_4$/PAGE. Fluorographs were exposed for 12 hr.

would be expected to reduce the size of the nascent protein by cleavage of the amino-terminal signal peptide (−1900) and the carboxyl-terminal signal peptide (−3000). However, glycosylation and PI-G tailing would be expected to increase the size of the processed protein. The molecular weight actually observed for processed PLAP synthesized *in vitro* was 67,100. Microsomes prepared from CHO cells grown in the presence of tunicamycin failed to produce the larger product, indicating that glycosylation was largely responsible for the observed increase in molecular weight (data not shown).

The amount of preproPLAP processed to the M_r 67,100 form was found to be dependent on the amount of microsomal membranes present during incubation; increasing amounts of microsomes resulted in a larger proportion of processed product (Fig. 4). However, microsomal membranes also inhibited the translation of preproPLAP. Therefore, the total amount of PLAP (processed and unprocessed) was found to be inversely proportional to the amount of membranes added (Fig. 4). Under the conditions used for translation, processing was dependent on the time of addition of the membranes to the reaction and thus was a cotranslational event (Fig. 5). Despite the inhibitory effects of microsomes on translation, the most efficient processing occurred when membranes were added at the start of the translation process and fell to 20% processing when translation had proceeded for 15 min before the addition of microsomes.

The addition of microsomes resulted in a product that not only exhibited a different molecular weight but also was sequestered inside the microsomal membranes. This was shown by resistance of the processed PLAP to protease digestion in the absence, but not in the presence, of detergent (Fig. 6, lane 5 vs. lane 6). Most of the processed PLAP (75–85%) was found to be inaccessible to protease. On the other hand, all of the preproPLAP was proteolyzed, indicating that it remained on the external side of the microsomes and thus was accessible to the protease, even in the absence of detergent (Fig. 6, lanes 2 and 3).

Translation of preproPLAP mRNA proceeded quite efficiently in the prokaryotic system and the product was similar to that produced in the eukaryotic system in size and interaction with the various antibodies (data not shown). However, attempts to have this product processed by microsomes have not been successful thus far, even with respect to demonstrating amino signal peptidase action and glycosylation.

Evidence for Amino-Terminal and Carboxyl-Terminal Cleavage During Processing. From the structures of preproPLAP and mature PLAP, it is evident that processing involves peptide cleavage at both the amino and carboxyl

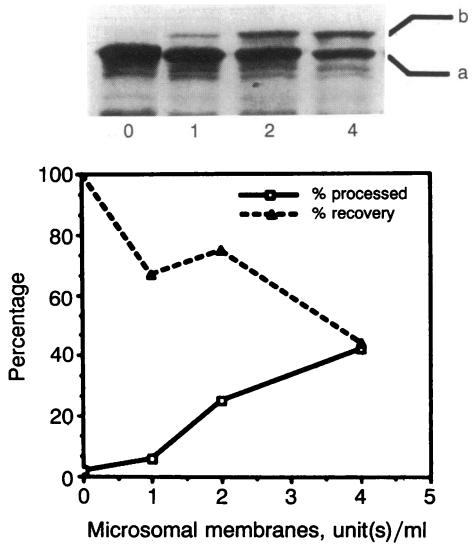


FIG. 4. Dependence of processing on microsomal membrane concentration. *In vitro* translation was carried out in the absence (lane 0) or presence (lanes 1, 2, and 4) of increasing amounts of CHO microsomal membranes. Lanes: 1, 3.5 units of microsomal membranes per ml; 2, 7.0 units/ml; 4, 14 units/ml—all added to the translation at the start of the reaction. Incubation was for 90 min at 30°C. After translation and electrophoretic separation of the [^{35}S]-methionine-labeled products, bands were located by autoradiography and excised from dried gels. Each band was rehydrated in distilled water and solubilized in 9:1 (vol/vol) NCS/H_2O (Amersham) for 2 hr at 55°C. Scintillant (Hydrofluor, National Diagnostics, Manville, NJ) was added, and the samples were assayed for radioactivity. Total ^{35}S label determined in the product in the absence of microsomes (lane 0, band a) was 8147 cpm. This was arbitrarily taken to represent 100% translation. Recovery was then calculated to be total cpm (bands a + b)/8147 × 100. The processed fraction (%) for samples translated in the presence of membranes was calculated from the ratio cpm in band b/total cpm (bands a + b). Processing in the absence of membranes was 0%.

termini. To determine whether such cleavage had occurred in the material processed by microsomes, the three site-specific antibodies were used. Changes in site-specific antibody reactivity were monitored relative to polyclonal anti-PLAP IgG, for which immunoreactivity remained constant in the presence and absence of microsomes. Evidence for processing at the amino terminus of nascent PLAP was monitored by immunoprecipitation with antibody directed to the amino terminus of mature PLAP (amino-Ab). Treatment of preproPLAP with amino-Ab yielded little immunoprecipitate. However, when microsomes were labeled during translation, the amount of PLAP immunoprecipitated with amino-Ab increased markedly (Fig. 7 *Upper*, lane 4, and *Lower*). Apparently cleavage of the amino-terminal signal peptide by signal peptidase frees a previously blocked internal epitope (Fig. 2) for reaction with the antibody. The specificity of the amino-Ab indicates that microsomal processing had cleaved preproPLAP to yield the amino terminus of the mature protein.

To determine whether correct processing had occurred at the carboxyl terminus, two separate site-specific antibodies, exo-Ab and endo-Ab (Fig. 2), were used, and their reactivities were compared to that of the polyclonal antibody. In a representative experiment comparing the reactivities of all three antibodies on the products from a single microsomal preparation, exo-Ab reactivity to the processed product formed in the presence of microsomal membranes decreased to 40% of its reactivity with preproPLAP (Fig. 7 *Upper*, lane 2, and *Lower*). Quantitatively, CHO microsomal preparations varied in their ability to cleave at the carboxyl terminus of preproPLAP from 20% to 90% efficiency (data not shown).

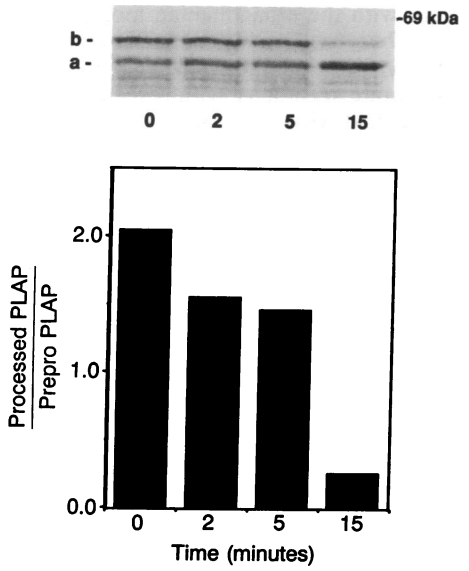


FIG. 5. Effect on processing of the time of addition of membranes to the translation reaction. *In vitro* translation was carried out in the presence of 5.7 units of CHO microsomal membranes per ml, which was added at the start of the translation reaction (lane 0) or 2, 5, or 15 min after the onset of translation. Total translation time in the presence of microsomes was 60 min at 30°C in all samples. The amount of preproPLAP processed was determined by densitometry of an autoradiograph obtained after electrophoretic separation of [^{35}S]methionine-labeled PLAP.

Thus, a substantial proportion of processed protein loses the exo-epitope as predicted by the model for carboxyl-terminal processing (Fig. 1). Furthermore, the endo-Ab reactivity of the product produced in the presence of microsomes remained the same as for preproPLAP, indicating that this epitope in the protein remains intact during processing (Fig. 7 *Upper*, lanes 1 and 3, and *Lower*). Based on a Hopp index plot of antigenic sites (13), the sequence used to generate the endo-Ab would be expected to show little difference whether internalized as in preproPLAP or at the carboxyl terminus as in mature PLAP.

These antibody reactivities do not eliminate the possibility that carboxyl-terminal cleavage was due to nonspecific proteolytic enzymes present in the microsomal preparations. However, the formation of a distinct product as shown by a sharp band on gels rather than a population of products, as well as the maintenance of a relatively constant amount of

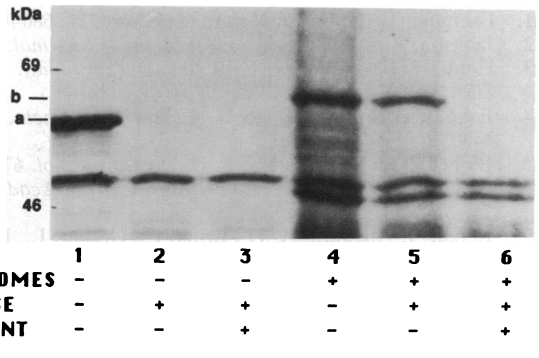
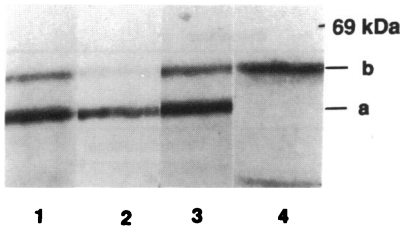


FIG. 6. Sequestration of processed product within microsomal vesicles. *In vitro* translation reactions were run in the absence (lanes 1–3) or presence (lanes 4–6) of 7.8 units of CHO microsomes per ml, which was added 2 min after the onset of translation. After translation for 60 min at 30°C, samples were treated with proteinase K as described. Fluorography was performed on [^{35}S]methionine-labeled PLAP separated on 7.5% acrylamide gels. Film exposure was for 12 hr at −70°C.



RELATIVE REACTIVITY AFTER PROCESSING BY MICROSOMES

Site-directed Antibody (Ab_S)	preproPLAP (a)	processed PLAP (b)
Exo-Ab	0.8	0.4
Endo-Ab	0.9	1.0
Amino-Ab	1.7	30.7

FIG. 7. Reactivities of site-directed antibodies with the product formed in the presence of CHO microsomes. (*Upper*) Immunoprecipitates were obtained and analyzed by $NaDodSO_4$/PAGE using polyclonal anti-PLAP IgG (lane 1), exo-Ab (lane 2), endo-Ab (lane 3), and amino-Ab (lane 4). Densitometry was then performed on the fluorographs of [^{35}S]methionine-labeled PLAP. (*Lower*) Each of the site-directed antibodies (Ab_S) exhibited a characteristic, reproducible, specific reactivity to nascent PLAP produced in the absence of microsomes. The ratio of this reactivity to that of the polyclonal antibody (Ab_{PC}) was determined: Ab_S reactivity/Ab_{PC} reactivity = reactivity ratio. This ratio was arbitrarily set at 1.0 for each Ab_S in nascent preproPLAP (unprocessed PLAP). An equivalent ratio was then determined for each Ab_S in the processed protein formed in the presence of CHO microsomes. The relative reactivity of the Ab_S = Ab_S reactivity after processing/Ab_S reactivity to nascent PLAP.

endo-epitope in the presence and absence of microsomes, argues strongly against this possibility. Further evidence for specific carboxyl-terminal cleavage rather than non-specific proteolysis is provided by the fact that all of the reactions were carried out in the presence of a cocktail containing several protease inhibitors. These did not affect cleavage of the exo-epitope. In addition to the inhibitors present in the cocktail, phenylmethylsulfonyl fluoride, *N*-tosyl-L-phenylalanine chloromethyl ketone, benzamidine, and α_2-macroglobulin were also found to be noninhibitory.

Attempts to Demonstrate Incorporation of Elements of the PI-G During *in Vitro* Processing. Based on information from earlier studies carried out *in vivo* on the incorporation of radiolabeled elements of the PI-G moiety into PLAP (2), we used large amounts of radiolabeled precursors in the present studies. When 50 μCi of [^{32}P]orthophosphate (9131 Ci/mmol, Amersham) was included in an incubation mixture containing microsomes, ≈10 fmol of processed PLAP was isolated. Based on the specific activity of [^{35}S]methionine-labeled product synthesized concurrently, if radiolabeled phosphate had been incorporated undiluted, we should have found 80,000 cpm in the immunoprecipitate obtained with polyclonal anti-PLAP antibody, assuming each molecule of PLAP contained a single phosphate in the PI-G tail. Furthermore, incorporation of label into as little as 1–2% of synthesized PLAP would have resulted in a detectable signal. We observed only background level counts. Experiments were also conducted by metabolic labeling of precursor(s) *in vivo* prior to preparation of microsomal membranes (14). When CHO cells were labeled *in vivo* with [^{32}P]orthophosphate (100 μCi per 2 × 10^6 cells) each day for 3 days and the microsomal fraction was harvested, it was found to contain 115,800 cpm. However, after incubation of an aliquot of prelabeled microsomes containing 77,000 cpm with the translation system, the PLAP synthesized *in vitro* contained <100 cpm over background. In other experiments similarly designed to

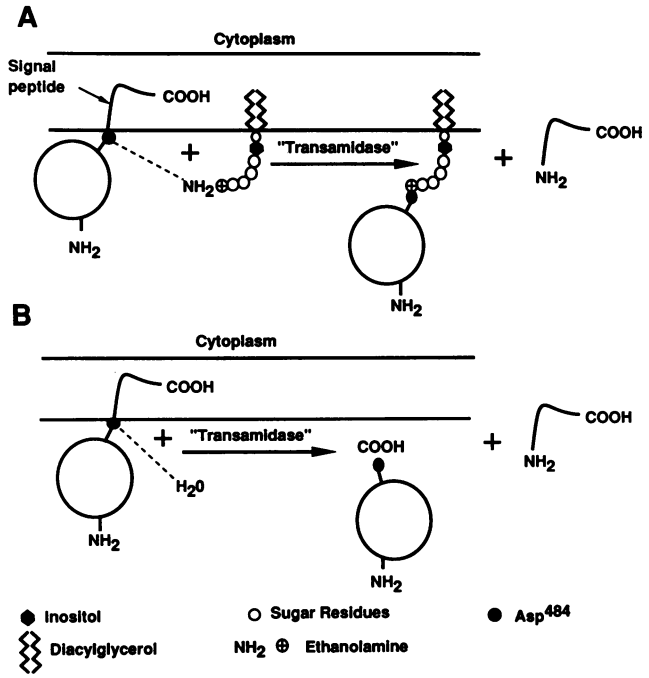


FIG. 8. Suggested mechanisms for the cleavage of the carboxyl-terminal signal peptide and addition of the PI-G tail. (*A*) Mechanism in the presence of a suitable substrate for PI-G tail addition. (*B*) Mechanism in the absence of a suitable endogenous PI-G precursor where water substitutes as a substrate.

measure incorporation of radiolabeled [^{32}P]ATP, [^{3}H]inositol, [^{3}H]ethanolamine, or [^{3}H]myristate/palmitate into PLAP synthesized and processed *in vitro*, no incorporation of label was detected. It should be noted that, except for the ^{32}P experiments, the ability to detect radiolabeled product would have been marginal even if the processing reaction used the possible precursors with close to 100% efficiency. This is due to the relatively low specific activity of the radiolabeled precursors.

In addition to the evidence presented here for correct processing at the carboxyl terminus of nascent PLAP, CHO microsomes are capable of other types of processing as well. Because of the specificity of the amino-terminal antibody to mature PLAP, the unmasking of the amino-terminal epitope after treatment with CHO microsomes indicates processing by amino-terminal signal peptidase (15). The microsomes also are capable of N-glycosylation (16). That these modifications are not random is shown by the appearance of essentially one product by NaDodSO$_4$/PAGE. The specificities of the two antibodies, endo-Ab and exo-Ab and their interactions with the translation product before and after addition of CHO microsomes further indicate that microsomes also cleave the carboxyl terminus in a nonrandom manner. Again, essentially one product appears whose interaction with polyclonal and endo-Abs is unchanged and is the same as that obtained with either nascent PLAP or PLAP isolated from placental tissue. However, the epitope for the exo-Ab has been lost, indicating cleavage of a carboxyl-terminal peptide.

The failure to obtain evidence for the incorporation of the elements of PI-G could be interpreted to mean that the carboxyl-terminal cleavage is due to the activity of a peptidase. It is conceivable that the overall process may start with the action of a specific peptidase, which is then followed by condensation of the newly exposed carboxyl terminus in amide linkage with the ethanolamine moiety of PI-G. This seems unlikely because formation of a biological amide bond would require a source of energy and also might be expected to exhibit substrate specificity with respect to the carboxyl-terminal amino acid. However, at least four different amino acid residues are now known to be acceptors for PI-G-tailing (5, 17). It is more likely that the amide linkage between the ethanolamine for PI-G and proPLAP is catalyzed by a transamidase (or transpeptidase) type of enzyme as shown in Fig. 8*A*, analogous to the actions of transglutaminase (18) or γ-glutamyl transpeptidase (19). It is possible that the correct donor of the amide nitrogen either was not produced in our *in vitro* preparations or, if produced, was not sufficiently labeled with radioisotope to be detected in the product. In the absence of an appropriate amide donor, a transamidase or transpeptidase can act as a hydrolase and react with water as shown in Fig. 8*B* (18–20).

Whether the observed carboxyl-terminal processing of nascent PLAP proceeds via the actions of a specific transamidase or of a peptidase cannot yet be determined. However, the procedures reported here should make it possible to monitor the isolation and purification of the enzyme(s) that catalyzes the process. Purified substrates and enzymes will permit obtaining unequivocal proof of the mechanism for the addition of the PI-G tail.

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