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Biochemistry

Aspartic acid-484 of nascent placental alkaline phosphatase condenses with a phosphatidylinositol glycan to become the carboxyl terminus of the mature enzyme

(cDNA/ethanolamine/peptide sequencing)

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ABSTRACT **A carboxyl-terminal chymotryptic peptide from mature human placental alkaline phosphatase was purified by HPLC and monitored by a specific RIA. Sequencing and amino acid assay showed that the carboxyl terminus of the peptide was aspartic acid, representing residue 484 of the proenzyme as deduced from the corresponding cDNA. Further analysis of the peptide showed it to be a peptidoglycan containing one residue of ethanolamine, one residue of glucosamine, and two residues of neutral hexose. The inositol glycan is apparently linked to the α carboxyl group of the aspartic acid through the ethanolamine. Location of the inositol glycan on Asp-484 of the proenzyme indicates that a 29-residue peptide is cleaved from the nascent protein during the post-translational condensation with the phosphatidylinositol-glycan.**

Membrane proteins vary greatly in the nature and extent of their interactions with the lipid bilayer. A number of diverse cell-surface proteins are anchored in plasma membranes by a phosphatidylinositol-glycan (PI-G) structure that is covalently attached to the carboxyl-terminal amino acid of the mature protein (1–3). Alkaline phosphatase [AP; nonspecific octophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] has been identified as a PI-G-tailed protein (4, 5). The mature enzyme, which is widely distributed in mammalian tissues, can be released from cellular membranes by phosphatidylinositol-specific phospholipase C (6, 7). Studies of AP, biosynthetically radiolabeled in cell culture with components of the putative PI-G moiety (8, 9), further support the initial classification of AP as a PI-G-tailed membrane protein. In higher primates and in man three isozymes of AP are present—namely, intestinal, placental, and the tissue-unspecific form present in liver, bone, kidney, and most other tissues (10, 11). The AP isozymes are highly glycosylated homodimers that have subunit molecular masses ranging from 60 to 80 kDa. The cDNA sequences of all three major types of mammalian AP have been deduced (12–17), and they all indicate the presence of a stretch of ≈20 hydrophobic amino acid residues at the carboxyl terminus of the nascent protein. It is believed, by analogy to the two best understood PI-G-tailed proteins, variant surface glycoprotein of *Trypanosoma brucei* (18–22) and Thy-1 antigen (23–26), that after synthesis on membrane-bound ribosomes the nascent form of AP is modified by (*i*) removal of an amino-terminal signal sequence, (*ii*) addition of N-linked oligosaccharides, (*iii*) replacement of a carboxyl-terminal hydrophobic peptide extension with a PI-G moiety that serves as a membrane anchor. The length of the carboxyl-terminal peptide that is removed from the nascent form of AP and the exact site of PI-G attachment, presumably via an amide bond between the amino group of ethanolamine and the carboxyl group of the carboxyl-terminal amino acid, are not known. To elucidate the post-translational modification of PLAP at its carboxyl terminus, we have purified the carboxyl-terminal chymotryptic peptide of mature human placental AP (PLAP). The peptide was characterized, and the carboxyl-terminal residue of mature PLAP was determined. Also, partial chemical characterization of the inositol-glycan anchoring structure is reported.

MATERIALS AND METHODS

Partially purified PLAP was purchased from Calbiochem. The enzyme came in a 50% (vol/vol) glycerol solution with a specific activity of 155.5 units/mg and a purity of 7.8%, calculated according to the standard set by Ezra *et al.* (27). Prior to denaturation, the enzyme was removed from glycerol and concentrated in Centriprep-10 concentrators (M_r, 10,000 cutoff membrane; Amicon) by centrifugation for 1 hr at 1000 × *g*. The enzyme was used without further purification.

Protein was measured by the Bio-Rad protein assay kit, a procedure originally described by Bradford (28). AP activity was asssayed at pH 9.8 in a microtiter plate in 1.0 M diethanolamine buffer by using *p*-nitrophenyl phosphate as a substrate according to the instructions in the Sigma catalog. One unit of enzyme activity corresponds to 1 μmol of substrate hydrolyzed per min at 37°C. The lipid content of PLAP was assayed by partitioning in a 0.5% Triton X-114 two-phase system as described by Malik and Low (29). α-Chymotrypsin, 7-amino-1-chloro-3-tosylamido-2-heptanone-treated type VII, was obtained from Sigma.

N-Ethylmorpholine (Fluka), dithiothreitol (Bethesda Research Laboratories), iodo[^{3}H]acetic acid (New England Nuclear), iodoacetic acid (Fluka), and Triton X-114 (Sigma) were used as received. 1-Propanol, acetic acid, and pyridine (Baker) were distilled over ninhydrin. All other chemicals were of analytical grade.

Reduction, carboxymethylation, and purification of the carboxymethylated PLAP were performed as described by Pan *et al.* (30). Reduced and carboxymethylated PLAP was subjected to chymotryptic digestion at 37°C for 24 hr with an amount of protease found to result in the maximal release of the cleavage products of interest (50 μg of chymotrypsin per

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Abbreviations: PI-G, phosphatidylinositol-glycan; AP, alkaline phosphatase; PLAP, placental AP; proPLAP, precursor of PLAP; RP-HPLC, reverse-phase HPLC.

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1000 μg of total protein, administered in three aliquots). The sample was then heated at 90°C for 30 min to inactivate the protease.

The strong anion-exchange HPLC Mono Q column (HR 5/5, 1 × 10 cm) was obtained from Pharmacia. Ion-exchange HPLC was performed with 20 mM *N*-ethylmorpholine (pH 6.8) as a buffer with a superimposed gradient of NaCl at a flow rate of 0.6 ml/min. The HPLC PartiSphere octadecyl column (4.6 mm × 110 mm, 5-μm particle size, 10-nm pores) was purchased from Whatman, and the HPLC μBondapack phenyl support (4.6 mm × 250 mm, 5-μm particles) was obtained from Waters Associates. Reverse-phase HPLC (RP-HPLC) was carried out in a 0.5 M pyridine/acetate buffer system, pH 4.0, with 1-propanol as the organic modifier at a flow rate of 0.5 ml/min. Three-minute fractions were collected. Postcolumn fluorescence detection of peptides utilized fluorescamine as described (31). ^{125}I-labeled [Met]enkephalin was used as the internal marker in RP-HPLC, and the ^{125}I-labeled PLAP haptene (see below) served the same purpose in the ion-exchange chromatography.

Chymotryptic peptides at the carboxyl terminus of PLAP were identified and assayed by means of a specific RIA that makes use of a polyclonal antiserum directed against residues 474–484 of the PLAP precursor (proPLAP). The antibody was produced by using the synthetic peptide Cys-Asp-Leu-Ala-Pro-Pro-Gly-Thr-Thr-Asp [proPLAP-(474–484)-undecapeptide] conjugated to keyhole limpet hemocyanin. The Cys-474 residue was used to link the haptene to hemocyanin through the sulfhydryl group (32). Tyrosine was coupled to the PLAP haptene through the sulfhydryl group to provide a site for labeling with ^{125}I. Details of the antiserum production in rabbits and the characteristics as well as the conditions of the RIA will be published. The site-directed antibody raised against PLAP-(474–484)-undecapeptide is highly specific, but its crossreactivity with the relevant chymotryptic PLAP peptide(s) ranged between 2.5 and 5%. An average value of 3% was used in all quantitations of PLAP-(474–484)-undecapeptide immunoreactive material. All RIAs reported in this paper were carried out after chymotryptic cleavage of reduced and carboxymethylated PLAP. In each experiment the amount of PLAP-(474–484)-undecapeptide immunoreactivity present in the unfractionated chymotryptic digest of PLAP was arbitrarily taken as 100%.

Amino acid, ethanolamine, and glucosamine analyses were performed by the postcolumn fluorescamine method as described (33). Neutral sugars were analyzed by a modification of the method described by Honda *et al.* (34). Microsequence analyses were performed on ≈300 pmol of the peptide by using an Applied Biosystems (Foster City, CA) model 470A protein sequencer similar to that described by Hewick *et al.* (35). Phenylthiohydantoin amino acid derivatives were identified "on-line" with an ABI model 120A phenylthiohydantoin analyzer or "off-line" with a Waters HPLC system with an Altex ultrasphere C_{18} column (4.6 mm × 25 cm) (36).

RESULTS

As mentioned above, with the sequences of the mature, PI-G-tailed proteins, variant surface glycoprotein and Thy-1, it was observed that they lack a carboxyl-terminal hydrophobic peptide extension of 17–31 amino acids predicted from their cDNA sequences (1–3). If the same were true in the case of PLAP, then the possible PI-G-tail attachment site, based on the sequence of proPLAP deduced from cDNA (13), would be somewhere in the region between Thr-483 and Val-493 (Fig. 1). Close examination of the hydropathy plot of the carboxyl-terminal region of proPLAP indicated a change from hydrophilic to a rather hydrophobic domain at Ser-491. That particular residue was considered to be one of the most likely candidates for the PI-G-attachment site. To monitor the purification of carboxyl-terminal proteolytic fragments of mature PLAP, a site-directed antibody was raised against proPLAP-(474–484)-undecapeptide with the belief that the most likely condensation site for the PI-G would be downstream from that region. We shall refer to proPLAP-(474–484)-undecapeptide as "endopeptide," for we know it to be still present within mature, membrane-bound PLAP.

Chymotrypsin was selected for proteolytic digestion of PLAP because its cleavage sites seemed most appropriate for obtaining a relatively small carboxyl-terminal fragment still attached to the inositol-glycan. The two chymotryptic cleavage sites that are closest to the possible carboxyl terminus of mature PI-G-tailed PLAP are Phe-464 and Tyr-471 (Fig. 1).

Prior to chymotryptic digestion, commercial PLAP was assayed for its diacylglycerol content by a Triton X-114 phase-separation method. The segregation of PLAP was measured in the detergent and aqueous phases by monitoring enzyme activity. More than 90% of commerical PLAP was found to partition in the aqueous phase, indicating that the diacylglycerol portion of the PI-G had been lost during preparation of the enzyme. The specificity of the RIA for PLAP endopeptide made it unnecessary to purify the commercial PLAP (≈8%) from contaminating proteins (≈92%). The amounts of enzyme taken for production of chymotryptic peptides were based on assay of PLAP enzyme activity. Several preliminary small scale experiments were carried out before the final large scale preparation. In the latter 27 nmol of unpurified PLAP was reduced, carboxymethylated, and treated with chymotrypsin. The data in Fig. 2 and Table 1 represent the results obtained on an aliquot of that digest representing 5% or 1.4 nmol. The remaining 95% was divided into several aliquots, each 15–20% of the total, and was purified over the same series of columns in several preparations. The resolutions and yields at each step of the purification were essentially the same for each of the large scale chromatographies as obtained with the 1.4-nmol aliquot.

As shown in Fig. 2 *Top*, chymotryptic peptides were initially separated on a RP-HPLC C_{18} column. RIA for endopeptide revealed two peaks of immunoreactivity eluting close to each other at 114 min and 120–123 min, respectively. Analyzing the retention times of the two peptides, one can clearly see that they are hydrophilic in nature, since they were eluted at a rather low concentration of 1-propanol in the mobile phase. This is in agreement with our preliminary purification attempts that indicated that the endopeptide crossreacting material from the total chymotryptic digest of mature PLAP had a substantial hydrophilic character as judged by the column supports with which it successfully

463 470 480 490 500 510 513

---AFAACLEPYTA CDLAPPAGTTD AAHPGRSVVPALLPLLAGTLLLLETATAP

Endo peptide sequence which recognizes the Endo Ab

Most likely condensation site for PI-G

FIG. 1. Carboxyl terminus of proPLAP. The single-letter amino acid code is used. Ab, antibody.

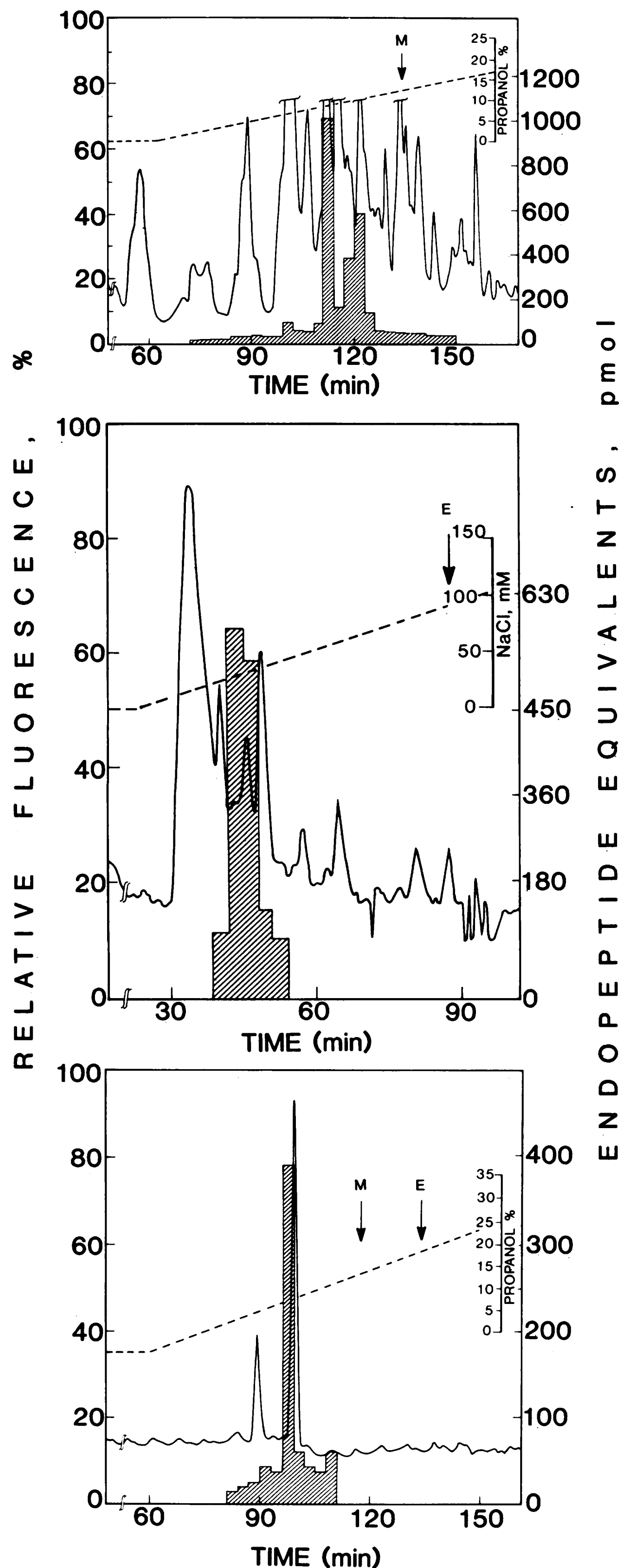

FIG. 2. Purification of the immunoreactive PLAP chymotryptic peptide. (*Top*) Chromatography of the chymotryptic digest on an RP-HPLC C_{18} column. (*Middle*) Chromatography of peak I from the C_{18} column on a Mono Q column. (*Bottom*) Chromatography of the first endopeptide immunoreactive fraction (peak I) from the Mono Q chromatography on an RP-HPLC phenyl column. The arrows indicate elution positions of internal standards, ^{125}I-labeled [Met]enkephalin (M) and ^{125}I-labeled endopeptide (E).

Table 1. Purification of carboxyl-terminal chymotryptic peptides of PLAP

Step	Endopeptide equivalents, pmol	% of total immunoreactivity
Chymotryptic digest	1400	100*
RP-HPLC C_{18}		
Peak I	924	66
Peak II	795†	56
Ion-exchange Mono Q, peak I	840	60
RP-HPLC phenyl, peak I	756‡	54

Peak I and II refer to the material eluting at 114 min and 120–123 min, respectively, in Fig. 2 *Top*.
*Arbitrarily taken as 100%.
†Peak II was not purified further in this experiment.
‡Endopeptide immunoreactivity appeared in two fractions in Fig. 2 *Middle*. Only the first one was used for the phenyl RP-HPLC shown in Fig. 2 *Bottom*. The second fraction was run independently (data not shown). The value represents the sum of endopeptide reactivity in the two fractions.

interacted. The fact that two endopeptide immunoreactive peaks were observed indicates that in this experiment chymotryptic cleavage did not proceed to completion. A single peak of immunoreactivity, eluting at the position of peak I was obtained when smaller amounts of PLAP were treated with chymotrypsin.

The peak eluted at 114 min (peak I) was concentrated *in vacuo* and rechromatographed on a Mono Q column. The pH was adjusted to 6.8 so that negatively charged polar side chains of acidic amino acids would remain in the ionized form (Fig. 2 *Middle*). The endopeptide immunoreactive material, was eluted rather early at 45–48 min, implying that it was not strongly acidic. One of the two fractions in which activity appeared (≈50%) was concentrated *in vacuo* and rechromatographed on a phenyl HPLC column. A sharp, symmetrical peak was obtained at 99–102 min, indicative of a single peptide species (Fig. 2 *Bottom*). Table 1 summarizes the stages of purification and yields obtained at each stage of the 5% preparation. The yield of highly purified peak I from the remaining 95% was ≈10 nmol estimated by endopeptide RIA and amino acid analysis (see below).

Aliquots of 300 pmol when subjected to sequence analysis revealed a tridecapeptide (Fig. 3). This sequence corresponds to residues 472–484 of proPLAP. It should be noted that in each of the three sequence determinations yields at the thirteenth cycle were sufficiently high so that Ala-485 of proPLAP, if present, would have been detected in cycle 14. These findings indicate that Asp-484 of proPLAP is the site of attachment of the PI-G group. Amino acid analyses (Table 2) were in accord with the sequencing data. Consistent with Asp-484 being the normal site of the attachment to the PI-G moiety the tridecapeptide was also shown to contain one equivalent each of ethanolamine and glucosamine (Table 2). Hydrolysates of the tridecapeptide also contained more than one residue of neutral sugar.

DISCUSSION

Our data show unequivocally that the carboxyl terminus of mature PLAP is an aspartic acid residue to which a PI-G moiety is attached, presumably through the α-carboxyl

TACDLAPPAGTTD~

FIG. 3. Sequence of the purified carboxyl-terminal PLAP chymotryptic peptide, indicating attachment to PI-G by ~. The single-letter amino acid code is used.

Table 2. Composition of PLAP carboxyl-terminal peptide

Component	Residues, no. per mol	
	Experimental*	Theoretical
Cysteine†	0.9	1
Aspartic acid	2.2	2
Threonine	2.4	3
Serine	0.2	0
Proline	2.4	2
Glycine	1.1	1
Alanine	2.9	3
Leucine	1.0	1
Ethanolamine	1.3	1
Glucosamine	0.9	1
Neutral hexose‡	1.6	?

For assay of amino acids and ethanolamine, 500-pmol samples were hydrolyzed in 6 M HCl containing 0.1% thioglycolic acid. Hydrolysis was carried out in vacuum-sealed ampules at 110°C. Amino acid assay required 24 hr for hydrolysis, and ethanolamine assay required 12 hr. For glucosamine assay 4 M HCl was used, and samples were hydrolyzed for 4 hr. For neutral sugars, samples were dissolved in 2 M trifluoroacetic acid, sealed under vacuum and heated at 100°C for 6 hr.
*Value represents the average of two independent analyses.
†Assayed as carboxymethyl cysteine.
‡Values are presented as neutral hexose, because the HPLC system that was used could not resolve mannose and galactose.

group, to the amino group of an ethanolamine. The aspartic acid residue is Asp-484 of proPLAP, indicating that a 29-residue peptide (Fig. 1) is cleaved out during the PI-G-tailing process. The 29-residue peptide is highly hydrophobic, as is true with other nascent forms of PI-G-tailed proteins. Its purpose may be to temporarily anchor proPLAP to the endoplasmic reticulum and to enable the cotranslational and posttranslational processing to occur.

Attachment of the PI-G moiety to the carboxyl terminus of mature PLAP partially explains its low crossreactivity with the endopeptide antiserum. The Asp-484 also represents the carboxyl terminus of the synthetic peptide used as a haptene. Since the peptide haptene was attached to the carrier protein through the cysteine at its amino terminus, Asp-484 would be expected to form an important immunodeterminant. Condensation of the aspartic acid with the inositol-glycan would be expected to interfere with binding to the endopeptide antibody. It was a fortunate coincidence that the PI-G was attached to the Asp-484. Had it been found attached any further upstream towards the amino terminus (e.g., to Thr-483 or Thr-482 of proPLAP) it is conceivable that the endopeptide antibody would not have been able to detect the PI-G-tailed carboxyl-terminal portion of PLAP.

The presence of more than one immunoreactive peptide after treatment with chymotrypsin (Fig. 2 *Top*) resulted from incomplete proteolysis, which seemed to occur when incubation mixtures were scaled up. The more hydrophobic chymotryptic peptide (peak II, 120–123 min) was isolated from other hydrolysates and shown to have the sequence Ala-Ala-Cys-Leu-Glu-Pro-Tyr-Thr-Ala-Cys-Asp-Leu-Ala-Pro-Pro-Ala-Gly-Thr-Thr-Asp. Proteolytic cleavage, therefore, occurred at Phe-464 (Fig. 1). No other immunoreactive peptides were observed in any experiments, indicating that the adjacent Asp-475 impaired cleavage at Leu-474, a possible chymotryptic cleavage site. It should be noted that several preparations were carried through the overall purification procedure that yielded sufficient material for sequencing. Although the amino terminus of the isolated immunoreactive peptides were sometimes alanine and sometimes threonine residues, in all cases the carboxyl terminus was an aspartic acid residue.

Inositol determinations are not reported here. Otherwise, the chemical analyses showed the expected components of the PI-G-anchoring mechanism (ethanolamine, a glycan of unidentified size, and glucosamine) with the exception of diacylglycerol, which had been lost during isolation of PLAP from the tissue. The presence of *myo*-inositol, ethanolamine, myristate, and palmitate in the PI-G moiety has been demonstrated in experiments involving biosynthetic labeling of PLAP in WISH (8) and HeLa (9) cells. However, the precise chemical composition of the PI-G structure in PLAP remains to be determined.

As this manuscript was being readied for submission we became aware of a presentation by Y. Ikehara,§ in which he suggested that Asp-484 is the site of attachment of PI-G to proPLAP.

§Ikehara, Y., Symposium on Posttranslational Modification of Proteins by Lipids, Les Diablerets, Switzerland, September 8–11, 1987, abstr.

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