

Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase

(signal peptide/COOH-terminal processing)

RADMILA MICANOVIC, LOUISE D. GERBER, JOEL BERGER*, KRISHNA KODUKULA, AND SIDNEY UDEFRIEND†

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

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ABSTRACT Many proteins are now known to be anchored to the plasma membrane by a phosphatidylinositol-glycan (PI-G) moiety that is attached to their COOH termini. Placental alkaline phosphatase (PLAP) has been used as a model for investigating mechanisms involved in the COOH-terminal processing of PI-G-tailed proteins. The COOH-terminal domain of pre-pro-PLAP provides a signal for processing during which a largely hydrophobic 29-residue COOH-terminal peptide is removed, and the PI-G moiety is added to the newly exposed Asp-484 terminus. This cleavage/attachment site was subjected to an almost saturation mutagenesis, and the enzymatic activities, COOH-terminal processing, and cellular localizations of the various mutant PLAP forms were determined. Substitution of Asp-484 by glycine, alanine, cysteine, asparagine, or serine (category I) resulted in PI-G-tailed and enzymatically active proteins. However, not all category I mutant proteins were PI-G tailed to the same extent. Pre-pro-PLAP with other substituents at position 484 (threonine, proline, methionine, valine, leucine, tyrosine, tryptophan, lysine, glutamic acid, and glutamine; category II) were expressed, as well as the category I amino acids, but there was little or no processing to the PI-G-tailed form, and this latter group exhibited very low enzyme activity. The bulk of the PLAP protein produced by category II mutants and some produced by category I mutants were sequestered within the cell, apparently in the endoplasmic reticulum (ER). Most likely, certain amino acids at residue 484 are preferred because they yield better substrates for the putative "transamidating" enzyme. In transfected COS cells, at least, posttranslational PI-G-tail processing does not go to completion even for preferred substrates. Apparently PI-G tailing is a requisite for transport from the ER and for PLAP enzyme activity. Proteins that are not transamidated are apparently retained in the ER in an inactive conformation.

Over 40 functionally diverse cell-surface proteins are now known to be anchored to the plasma membrane of eukaryotic cells by covalent linkage to a phosphatidylinositol-glycan (PI-G) moiety in the outer leaflet of the lipid bilayer (1, 2). The "PI-G tail" is added to an internal residue of the nascent protein with the concomitant removal of a COOH-terminal signal peptide of ≈ 17 –31, mainly hydrophobic, residues (1, 2). This processing presumably takes place on the luminal side of the membrane of the rough endoplasmic reticulum (ER) (3, 4). The mature protein with the PI-G tail on the COOH-terminal residue is then presumably transported through the remaining elements of the intracellular transport system involved in the bulk flow of proteins to the cell surface (5).

Human placental alkaline phosphatase (PLAP) is a PI-G-tailed membrane glycoprotein that is stable and easily

assayed (6). It is, therefore, an excellent model for investigating the mechanisms involved in COOH-terminal processing and attachment of the PI-G anchor. PI-G-tailed membrane-bound PLAP can be overexpressed by COS cells transfected with a PLAP cDNA expression vector (7). COOH-terminal deletion of PLAP prepared to elucidate the structural requirements of the COOH-terminal signal peptide revealed that a hydrophobic domain of a certain minimal size is necessary for correct processing to occur (8). The exact primary sequence is of lesser importance. Similar conclusions were obtained in studies of another PI-G-tailed protein, decay accelerating factor (9). During processing of nascent PLAP, a 29-residue peptide is removed from the COOH terminus, and the PI-G moiety condenses with the newly exposed Asp-484 residue (Fig. 1) (10). Listed in Fig. 1 are proteins in which amino acids other than aspartic acid serve as sites of PI-G tailing. It was of interest to determine whether these amino acids (category I) when substituted for Asp-484 can serve as PI-G-tailing sites in PLAP and to survey other amino acids (category II) for this capability. An almost saturation mutagenesis of that position is reported here, indicating a certain selectivity of the cleavage/attachment site.

MATERIALS AND METHODS

Mutagenesis and Recombinant Plasmids. Asp-484 substitution mutants of wild-type PLAP (Asp-484) were prepared by oligonucleotide-directed mutagenesis of an M13mp19/PLAP vector as described originally by Zoller and Smith (17) and modified by Kunkel *et al.* (18) to ensure strong selection against wild-type clones. The vector was constructed by subcloning a 1.6-kilobase (kb) *Bam*HI–*Kpn*I fragment of PLAP cDNA into the corresponding restriction sites of the M13 multiple cloning site. All mutant clones were plaque-purified and verified by DNA sequencing (19). Two of the 484 mutants, proline and leucine, contained a second substitution at residue 487, glutamine for histidine, a conservative mutation. Upon M13 *in vitro* mutagenesis a 1.6-kb *Bam*HI–*Kpn*I PLAP cDNA fragment was recloned in the mammalian expression vector pBC12B1. The latter, which utilizes Rous sarcoma virus long terminal repeat as the promoter, has been described (7).

Cell Culture and DNA Transfections. COS-7 cells (20) were maintained in culture as described (21) and were transfected in culture trays containing six 35-mm wells, by the DEAE-dextran method (22).

Abbreviations: PI-G, phosphatidylinositol-glycan; PLAP, human placental alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum.

*Current address: Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065-0900.

†To whom reprint requests should be addressed at: Department of Neurosciences, Roche Institute of Molecular Biology, 340 Kingsland Street, Nutley, NJ 07110.

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-----P⁴⁸⁰AGTTD⁴⁸⁴A⁴⁹⁰AHPGR⁵⁰⁰SVVPALLP⁵¹⁰LLAGTLLLE⁵¹⁰TATAP

CATEGORY I

Asp	PLAP (10, 11)
Gly	Erythrocyte acetylcholinesterase (12)
Ala	Carcinoembryonic antigen (13)
Cys	Thy I (14)
Asn	<i>T. brucei</i> , VSG variant Group 3 MITat (15)
Ser	<i>T. brucei</i> , VSG variant Group 2 ILTat (16)

CATEGORY II

Thr	Trp
Pro	Tyr
Met	Lys
Val	Glu
Leu	Gln

Alkaline Phosphatase and Protein Assay. After transfection, the cell medium was removed and centrifuged for 2 min at $14,000 \times g$ in a microcentrifuge to remove cell debris. The cells were washed and homogenized as described (7). Media and cell homogenates were then assayed for alkaline phosphatase activity using *p*-nitrophenyl phosphate as substrate (23). One milliunit produces an absorbance change of 0.04 per min at 405 nm. The assay was made specific for PLAP activity by adding 10 mM (final concentration) L-homoarginine to inhibit traces of endogenous alkaline phosphatase in the COS cells (7). In certain experiments transfected COS cells were permeabilized with 0.1% Triton X-100 before assaying PLAP activity. Cell homogenates were treated with 0.1 M NaOH, and protein was determined by the method of Bradford (24).

Metabolic Labeling and Immunoprecipitation. Metabolic labeling of cells with [³⁵S]methionine (Amersham) was done 60 hr after transfection. For steady-state labeling, cells were preincubated for 1 hr in 2 ml of methionine-free Dulbecco's minimal essential medium (DMEM) supplemented with 5% dialyzed fetal calf serum (GIBCO). Fresh medium containing [³⁵S]methionine (1440 Ci/mmol, 200 μ Ci per 35-mm dish; 1 Ci = 37 GBq) was then added, and incubation was continued for 12 hr. Labeling with [³H]ethanolamine (Amersham; 200 μ Ci per 35-mm dish) was performed overnight in a 1:1 mixture of F-12 and DMEM supplemented with 10% dialyzed fetal calf serum. After radiolabeling, the culture medium was removed and cleared by centrifugation. Cells were harvested by scraping and lysed with 2% SDS/5% (vol/vol) 2-mercaptoethanol. Immunoprecipitation of labeled PLAP and analyses by SDS/PAGE followed by fluorography were performed as described (6). Proteins were immunoprecipitated with a 1000-fold dilution of a purified polyclonal antibody to PLAP (Dako, Santa Barbara, CA).

Treatment of Cells with Phospholipase C and Bromelain. PLAP was released from the surface of transfected COS cells with phosphatidylinositol-specific phospholipase C (PI-PLC) purified from *Bacillus thuringiensis* (25). Duplicate 35-mm wells of COS cells were transfected and grown in the presence of [³⁵S]methionine for each mutant studied. Each well was then washed twice with 2 ml of phosphate-buffered saline and twice with 2 ml of release buffer (25 mM Tris-HCl, pH 7.5/0.25 M sucrose/10 mM glucose). After this, 1 ml of release buffer containing 5 mM phenylmethylsulfonyl fluoride, 100 μ M *N*^α-*p*-tosyl-L-lysine chloromethylketone, 100 μ M leupeptin, and 5 mM iodoacetate was added to each well. For each mutant, 120 units of PI-PLC in 80 μ l was added to one well, and 80 μ l of 50 mM Tris-HCl, pH 7.5, was added to another as control. After incubation at 37°C for 2 hr supernatants were removed and centrifuged at $14,000 \times g$ for 5 min to remove cell debris. Aliquots were taken to measure PLAP activity or to immunoprecipitate for SDS/PAGE, and the remaining cells were washed twice with phosphate-buffered saline and then lysed with 400 μ l of 2% SDS/5% 2-mercaptoethanol. The lysates were immunoprecipitated and subjected to SDS/PAGE.

FIG. 1. The COOH terminus of PLAP with the signal peptides, which is removed during PI-G tailing, is shown in white type on black. Asp-484, the site of PI-G tailing, is the site of mutation in these studies. Amino acids found as sites of PI-G tailing in other proteins are listed below under category I. Numbers in parentheses are references. Category II amino acids are those that have not yet been found as sites for PI-G tailing. *T.*, *Trypanosoma*; VSG, variable surface glycoprotein.

Release of PLAP from intact COS cells by bromelain was performed in a manner similar to that described for PI-PLC, except that protease inhibitors were omitted and 0.3 unit of bromelain was included. After incubation bromelain activity was inhibited by 5 mM iodoacetate, and leupeptin and *N*^α-*p*-tosyl-L-lysine chloromethylketone were added to a final concentration of 100 μ M each. The cells that were treated with bromelain detached from the well and were removed by centrifugation at $14,000 \times g$ for 5 min. Controls were treated as in the PI-PLC experiments.

Immunofluorescent Labeling of Cells. Immunofluorescent labeling of intact or permeabilized cells was performed as described (26). For visualization of surface antigen, cells were incubated with anti-PLAP antibody diluted 1:500 in bovine serum albumin buffer (1% bovine serum albumin/0.3% gelatin/25 mM Na₂HPO₄, pH 7.5/0.15 M NaCl). For visualization of the intracellular antigen, the cells were incubated with the same antibody diluted 1:500 in Tween buffer (0.5 M NaCl/1% bovine serum albumin/5 mM NaH₂PO₄, pH 6.5/0.5% Tween 20). Cells were washed to remove excess PLAP antibodies and incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin antibodies (Boehringer Mannheim) diluted 1:50 in Tween buffer. Processed slides were examined in a fluorescence microscope.

RESULTS

Expression of Asp-484 Site-Directed Mutants of PLAP. All 16 PLAP Asp-484 site-directed mutant cDNAs that were prepared are summarized in Fig. 1. They were transiently expressed in COS cells as monitored by [³⁵S]methionine labeling followed by immunoprecipitation with anti-PLAP antibody. From several experiments similar to the one shown in Fig. 2 it became apparent that all Asp-484 substitution mutants were expressed to approximately the same extent, within experimental error, exhibited molecular masses (\approx 67 kDa) comparable to the wild-type PLAP 484, and were exclusively associated with the cells. Most mutants in category I exhibited as high or higher levels of enzyme activity than the wild-type Asp-484 PLAP (Table 1). Except for proline, category II mutants exhibited lower, but measurable, enzyme activity, particularly valine and leucine. Permeabilization of cells with 0.1% Triton X-100 did not affect enzyme activity, indicating that most activity was associated with the cell surface.

Evidence for PI-G Tailing of Mutants. Asp-484 PLAP can be released from the cell surface by treatment with PI-PLC (6, 7). To determine the nature of association of the mutant proteins to cells, [³⁵S]methionine-labeled COS cells were subjected to PI-PLC treatment. Aliquots of the cell extracts and media were immunoprecipitated with PLAP antibody, and radiolabeled PLAP proteins were resolved by SDS/PAGE. Fig. 3a shows only the release of selected mutant proteins from COS cells into the medium. Corresponding controls with no PI-PLC treatment showed virtually no release (data not shown). It is apparent that all the category I mutants were released by the lipase. PI-PLC also released

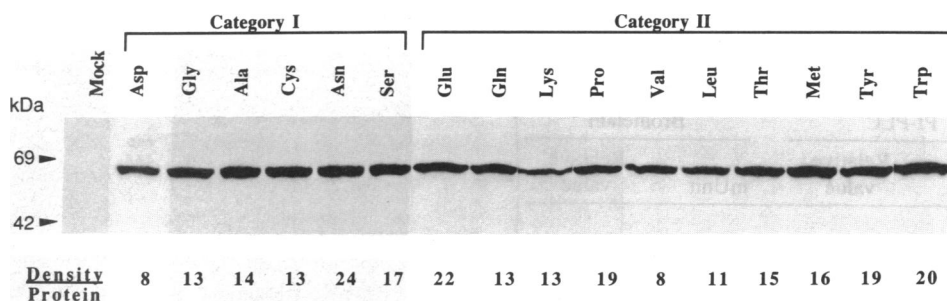


FIG. 2. Expression of PLAP encoded by wild-type (Asp-484) and mutant cDNAs in transfected COS cells. Transfections were done as described with constant amounts of PLAP cDNA. After transfection the cells were lysed, and labeled PLAP was immunoprecipitated and subjected to SDS/PAGE followed by fluorography. This result represents one of several such experiments. The observed differences in expression (densitometric measurements on the film; Density) among the mutants was, in part, due to random error and, to a large extent, the result of differences in plating, which yielded a different number of cells in each well. Cell protein was measured in each sample, and the ratio of density/protein was calculated to partly correct the observed variations. In this experiment aspartic acid and valine gave low values, and asparagine and tryptophan gave high values. However, in other experiments one or two amino acids other than aspartic acid and valine gave low values, and amino acids other than asparagine and tryptophan gave high values.

small amounts of PLAP protein from two category II mutants, valine and leucine, and trace amounts from some of the others. Although the data are not shown, residual ³⁵S-labeled PLAP in category I mutant cells after PI-PLC was diminished, whereas that in category II mutant cells was little changed. COS cells producing the various mutants were also treated with bromelain, which releases cell-surface membrane-bound PLAP by cleaving a peptide of ≈2 kDa from the COOH terminus (27). Bromelain released PLAP protein from the same mutant cells and in approximately the same proportions as did PI-PLC (Fig. 3*b*). Except for valine and leucine, and perhaps threonine, there was little detectable ³⁵S-labeled PLAP released from category II mutants.

Similar experiments were done monitoring PLAP enzyme activity instead of PLAP protein. As shown in Table 2 all mutant cells that yielded enzymatically active PLAP released enzyme activity when treated with either PI-PLC or bromelain. It should be noted that the enzyme activity released by either treatment was proportional to the amount of enzyme activity produced by that mutant (see Table 1). This is not surprising because in our experience 60–75% of enzyme activity is released from wild-type PLAP by PI-PLC and an even larger percentage by bromelain.

Table 1. PLAP enzyme activity in COS cells transfected with Asp-484 mutant cDNAs

Mutant	Category I		Mutant	Category II	
	mUnit/ μg of protein	Relative value		mUnit/ μg of protein	Relative value
Asp (wt)	1.45	1	Thr	0.07	0.06 ± 0.02
Gly	1.99	1.17 ± 0.16	Pro	0.00	0
Ala	1.45	1.02 ± 0.16	Met	0.06	0.04 ± 0.01
Cys	0.84	0.39 ± 0.16	Val	0.22	0.10 ± 0.03
Asn	2.58	2.70 ± 0.91	Leu	0.24	0.13 ± 0.01
Ser	3.08	2.59 ± 0.34	Trp	0.07	0.04 ± 0.01
			Tyr	0.05	0.05 ± 0.02
			Lys	0.04	0.04 ± 0.02
			Glu	0.06	0.05 ± 0.02
			Gln	0.06	0.04 ± 0.01

Values for mock transfections averaged about 0.02 milliunit (mUnit)/μg and were subtracted. The mUnit/μg of protein shown above for each mutant represent the average of duplicate analyses obtained in one experiment. Because of differences in transfection efficiency from experiment to experiment, these values could not be averaged; instead, the relative values obtained on three to four separate experiments are shown with aspartic acid arbitrarily taken as 1.0. Relative values shown represent the mean ± SEM. wt, Wild type.

As further evidence of PI-G tailing, COS cells transfected with representative mutants were incubated in the presence of [³H]ethanolamine, and the labeled proteins were isolated by immunoprecipitation. Ethanolamine is one of the components of the PI-G anchor of PLAP (6). Mutant proteins of category I incorporated far more [³H]ethanolamine than did those of category II (Fig. 4).

Cellular Localization of Enzymatically Inactive Mutant Proteins. To further investigate the nature of cellular association of the mutant proteins immunofluorescent staining of intact and permeabilized cells was performed. Data from representative examples of the PLAP mutants are shown in Fig. 5. Cells transfected with the mutants asparagine and serine, representatives of category I, yielded intense fluorescence before permeabilization, once again proving their cell-surface localization. By contrast, the mutants tryptophan and valine, representing category II, exhibited only faint fluorescence on intact cells, which was intensified after permeabilization. This is consistent with their largely intracellular localization, most probably in association with the membrane of the ER.

DISCUSSION

There exists an apparent similarity in specificities between COOH-terminal processing and NH₂-terminal signal peptidase. Both types of processing require a hydrophobic chain of comparable size (≈20–30 residues) either preceding or following the cleavage site. In both cases amino acids with small substituents on the β carbon atom are preferred at the

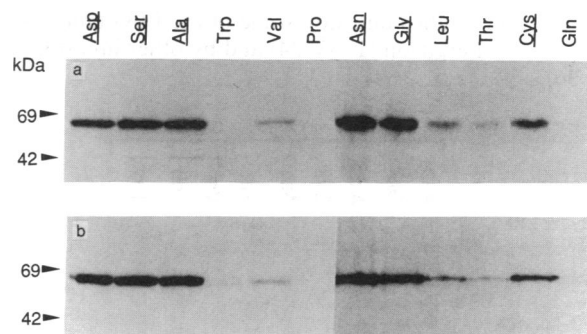


FIG. 3. Release of ³⁵S-labeled PLAP mutant proteins from COS cells by PI-PLC (a) and bromelain (b). Labeled PLAP in the supernatants was immunoprecipitated and subjected to SDS/PAGE and fluorography. Data from two experiments were combined. The first six mutant cells incubated with either PI-PLC or bromelain were run in one experiment; the last six mutant cells were run in another experiment. Category I mutants are underlined.

Table 2. Release of PLAP activity by PI-PLC and bromelain from COS cells transfected with different mutants

Mutant	PLAP activity released			
	PI-PLC		Bromelain	
	mUnit	Relative value	mUnit	Relative value
Category I				
Asp (wt)	80	1.0	90	1.0
Gly	64	0.83 ± 0.02	92	0.93 ± 0.08
Ala	92	0.93 ± 0.26	97	0.98 ± 0.12
Cys	24	0.27 ± 0.05	26	0.27 ± 0.02
Asn	170	2.18 ± 0.11	182	2.12 ± 0.28
Ser	245	2.56 ± 0.57	224	2.28 ± 0.26
Category II				
Thr	2	0.03 ± 0.02	4	0.04 ± 0.01
Pro	2	0.02	0	0
Met	1	0.01 ± 0.01	2	0.01 ± 0.01
Val	10	0.12 ± 0.01	6	0.07 ± 0.03
Leu	7	0.10 ± 0.01	10	0.10 ± 0.01
Trp	2	0.01 ± 0.01	1	0.03 ± 0.02
Gln	2	0.02 ± 0.02	2	0.01 ± 0.01

Blanks were run for each mutant and incubated for the same time but without enzyme. These ranged from 0 to 2 milliunits (mUnit) and were subtracted from the values obtained after enzyme treatment. The values for milliunits released are the average of duplicates from one typical experiment. Because the absolute amounts produced varied considerably from experiment to experiment the amounts released could not be averaged. However, the relative amounts released were similar in all experiments. Aspartic acid is arbitrarily taken as 1.0, and the relative values shown represent the mean ± SEM based on three to four separate experiments. Where no SEM is shown, too few replicates were taken for calculation.

COOH side of the site of cleavage as well as at the adjacent sites (1, 28, 29). A survey of NH₂-terminal cleavage sites (30) shows that glycine, alanine, serine, and occasionally cysteine are allowable. These are similar to the amino acids preferred for COOH-terminal processing. However, aspartic acid and asparagine have not been reported to serve as NH₂-terminal cleavage sites. In making such comparisons it is important to remember that NH₂-terminal signal processing is catalyzed by a peptidase (31), whereas COOH-terminal processing presumably requires a transamidase (32). The observed differences in activities of the various substituents at residue 484 will probably be explained by the requirement of a specific secondary structure at the cleavage/substitution site, such as has been shown for NH₂-terminal signal processing (28). It remains to be seen whether the specificities at the site of PI-G tailing seen here are the same in other cells and for other proteins. Our studies question some of the PI-G tailing sites predicted by Ferguson (1, 33, 34) and by other investigators (35, 36).

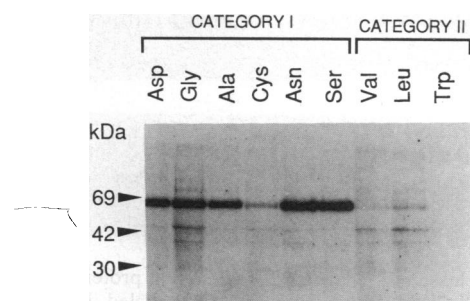


FIG. 4. Incorporation of ethanolamine into selected Asp-484 mutant PLAP proteins. Transfected COS cells were incubated with [³H]ethanolamine, and extracted proteins were immunoprecipitated and subjected to SDS/PAGE and fluorography.

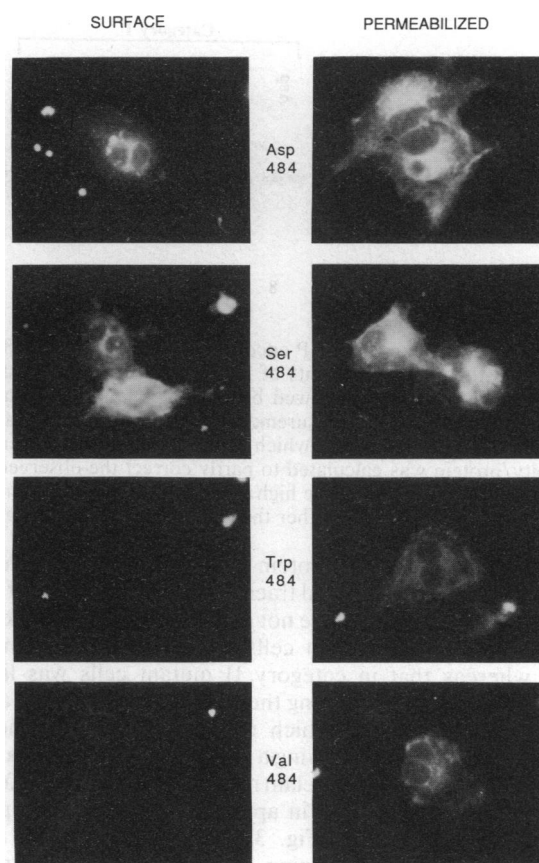


FIG. 5. Detection of wild-type and selected mutant proteins by immunofluorescent staining of transfected COS cells. Cells were fixed, and nonspecific binding sites were blocked with normal goat serum. Intact and permeabilized cells were treated with anti-PLAP antibodies, and bound antibody was visualized by staining with rhodamine-conjugated goat anti-rabbit immunoglobulin antibodies.

The present studies revealed unexpected complexities in the PI-G tailing process. Thus, all mutants were expressed to approximately the same extent—i.e., they yielded approximately the same amount of immunoprecipitable PLAP protein for the same amount of cDNA used for transfection. Because the apparent molecular masses of all mutant proteins were the same, all were apparently glycosylated to the same extent, indicating their processing in the ER. The first unexpected finding was that the enzyme activities resulting from the different substituents at position 484 varied considerably, from about 3.0 milliunits/μg of protein for asparagine and serine to 0.8 for cysteine and about 0.2 for valine and leucine. Except for proline, which yielded no activity, the other category II mutants produced lower but detectable PLAP enzyme activity. Furthermore, enzymatically active PLAP produced by all mutants was releasable from intact cells by PI-PLC and bromelain, indicating that the enzyme was on the cell surface and PI-G-tailed (Fig. 3 and Table 2). In addition, when cells were labeled with [³⁵S]methionine and treated with PI-PLC or bromelain, the relative amounts of ³⁵S label released from each mutant were comparable to the enzyme activity of that mutant (Fig. 3 and Table 2). Thus asparagine and serine with the highest enzyme activity, even higher than the wild-type Asp-484, released the largest amounts of labeled protein. The cysteine mutant released less, and the leucine and valine mutants released still less. Most other category II mutants produced too little enzyme activity to permit such experiments. Because each mutant produced comparable amounts of PLAP protein, the observed variations in the amounts of enzyme activity and in

PI-PLG- and bromelain-releasable material can only be explained by different degrees of PI-G tailing. That would mean that all mutants are PI-G-tailed to some extent and that PI-G tailing is a requisite for externalization, membrane attachment, and enzyme activity. It also suggests that all mutant proteins, including Asp-484, do not yield PLAP that is fully PI-G-tailed. When one compares the enzyme activity of Asp-484 to that of the serine mutant, it is <50%. Accordingly, nascent PLAP produced by Asp-484 should be only partially PI-G-tailed. In fact, experiments that have puzzled us for a long time suggest that this may well be the case. The availability of antiserum specific for the COOH terminus of nascent PLAP (exoAb) (37) permitted us to monitor the release of the 29-residue signal peptide. It was found that COS cells transfected with wild-type PLAP cDNA when lysed and subjected to immunoprecipitation with exoAb always tested highly positive for the COOH-terminal signal peptide. Furthermore, this label persisted in pulse-chase experiments, indicating that it did not represent a small amount of protein in the process of synthesis but a rather stable product. Details of these studies will be published elsewhere. It is conceivable that partial PI-G tailing is an artifact of transfection in COS cells, particularly because they are overproducing PLAP. However, we have also seen significant amounts of PLAP produced by WISH cells under standard culture conditions that still retain the COOH-terminal signal peptide (unpublished data).

One scenario that can explain the above findings is that the specificity of the putative transamidase that cleaves the protein and adds the PI-G tail (32) determines which substituents at residue 484 are preferable. The most preferred, such as serine and asparagine, being the best substrates, would be processed to the greatest extent, whereas proline, being the poorest substrate, may not be processed at all. That portion of each mutant protein that is not processed would lack the signal (apparently the PI-G tail) for transport to the Golgi apparatus and would be retained in the ER. As pointed out by Lodish (38), the conformations of aberrant proteins retained in the ER are altered. In the above scenario all PLAP mutants originally have conformations that can lead to enzyme activity but lose this potential during longer than usual residence in the ER. A second scenario to explain the present findings is that as the polypeptide chain grows, different substituents at residue 484 immediately lead to varying amounts of conformational change throughout the protein. Only that fraction of a given nascent protein that maintains its native conformation could then serve as substrate for the transamidase in the ER and therefore be transported to the Golgi apparatus; the remainder would be retained in the ER. There is one obvious argument against scenario 2. Presumably drastic structural changes would occur only if residue 484 were in some way involved in maintaining the overall (enzymatically active) conformation of PLAP. However, treatment of mature PLAP with bromelain, which cleaves ≈ 15 or more residues from the COOH terminus (27), has no effect on enzyme activity, indicating that residue 484 is not directly involved in achievement of the active conformation. There is a more direct way to distinguish between these two opposing scenarios. By deleting portions of the hydrophobic signal peptide from the COOH terminus of pre-PLAP, mature enzymatically active protein that is not PI-G-tailed has been produced that is secreted from the cell into the medium (8). By carrying out site-directed mutagenesis at Asp-484 of such secreted forms of PLAP one can determine whether substitution of the residues listed under category II mutants (Table 1) render the secreted PLAP enzymatically inactive and, therefore, folded incorrectly. If not, then the most likely explanation of our present findings is scenario

1—i.e., all 484 mutants produce nascent proteins with conformations suitable for further processing to active enzyme. However, certain amino acids at residue 484 are preferred to others as substrates for the "transamidating" enzyme (32). What is not transamidated is retained in the ER with subsequent loss of the favored conformation.

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- Ferguson, M. A. J. & Williams, A. F. (1988) *Annu. Rev. Biochem.* **57**, 285–320.
- Low, M. G. (1989) *FASEB J.* **3**, 1600–1608.
- Bangs, J. D., Hereld, D., Krakow, J. L., Hart, G. W. & Englund, P. T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3207–3211.
- Ferguson, M. A. J., Duzenko, M., Lamont, G. S., Overath, P. & Cross, G. A. M. (1986) *J. Biol. Chem.* **261**, 356–362.
- Pfeffer, R. S. & Rothman, J. E. (1987) *Annu. Rev. Biochem.* **56**, 829–852.
- Howard, A. D., Berger, J., Gerber, L., Familetti, P. & Udenfriend, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6055–6059.
- Berger, J., Howard, A. D., Gerber, L., Cullen, B. R. & Udenfriend, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4885–4889.
- Berger, J., Howard, A. D., Brink, L., Gerber, L., Hauber, J., Cullen, B. R. & Udenfriend, S. (1988) *J. Biol. Chem.* **263**, 10016–10021.
- Caras, I. W. & Weddell, G. N. (1989) *Science* **243**, 1196–1198.
- Micanovic, R., Bailey, C. A., Brink, L., Gerber, L., Pan, Y.-C. E., Hulmes, J. D. & Udenfriend, S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1398–1402.
- Ogata, S., Hayaishi, Y., Takami, N. & Ikehara, Y. (1988) *J. Biol. Chem.* **263**, 10489–10494.
- Haas, R., Brandt, P. T., Knight, J. & Rosenberry, T. L. (1986) *Biochemistry* **25**, 3098–3105.
- Hefta, S. A., Hefta, L. J. F., Lee, T. D., Paxton, R. J. & Shively, J. E. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4648–4652.
- Tse, A. G. D., Barclay, A. N., Watts, A. & Williams, A. F. (1985) *Science* **230**, 1003–1008.
- Boothroyd, J. C., Cross, G. A. M., Hoeijmakers, J. H. J. & Borst, P. (1980) *Nature (London)* **288**, 624–626.
- Majumder, H. K., Boothroyd, J. C. & Weber, H. (1981) *Nucleic Acids Res.* **9**, 4745–4753.
- Zoller, M. J. & Smith, M. (1982) *Nucleic Acids Res.* **10**, 6487–6500.
- Kunkel, T. A., Roberts, J. D. & Fabour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
- Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4767–4771.
- Gluzman, Y. (1981) *Cell* **23**, 175–182.
- Cullen, B. R. (1986) *Cell* **46**, 973–982.
- Cullen, B. R., Lomedico, P. T. & Ju, G. (1984) *Nature (London)* **307**, 241–245.
- McComb, R. B. & Bowers, G. N., Jr. (1972) *Clin. Chem.* **18**, 97–104.
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
- Taguchi, R., Asahi, Y. & Ikezawa, H. (1980) *Biochim. Biophys. Acta* **619**, 48–57.
- Cullen, B. R. (1987) *Methods Enzymol.* **152**, 684–703.
- Koffel, R. H. & Hanford, W. C. (1980) *J. Biochem. Biophys. Methods* **2**, 325–330.
- Pollitt, S., Inouye, S. & Inouye, M. (1986) *J. Biol. Chem.* **261**, 1835–1837.
- Wickner, T. W. & Lodish, F. H. (1985) *Science* **230**, 400–407.
- Von Heijne, G. (1983) *Eur. J. Biochem.* **133**, 17–21.
- Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
- Bailey, C. A., Gerber, L., Howard, A. D. & Udenfriend, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 22–26.
- Waneck, G. L., Sherman, D. H., Calvin, S., Allen, H. & Flavell, R. A. (1987) *J. Exp. Med.* **165**, 1358–1370.
- Prat, A., Katinka, M., Caron, F. & Meyer, E. (1986) *J. Mol. Biol.* **189**, 47–60.
- Waneck, G. L., Stein, E. M. & Flavell, R. A. (1988) *Science* **241**, 697–699.
- Caras, I. W., Weddell, G. N. & Williams, R. S. (1989) *J. Cell Biol.* **108**, 1387–1396.
- Bailey, C. A., Howard, A. D., Micanovic, R., Berger, J., Heimer, E., Felix, A., Gerber, L., Brink, L. & Udenfriend, S. (1988) *Anal. Biochem.* **170**, 532–541.
- Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 2107–2110.