Site-specific Mutations in the COOH-Terminus of Placental Alkaline Phosphatase: A Single Amino Acid Change Converts a Phosphatidylinositol-glycan–anchored Protein to a Secreted Protein

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Abstract. Placental alkaline phosphatase (PLAP) is anchored in the plasma membrane by a phosphatidylinositol-glycan moiety (PI-glycan). PI-glycan is added posttranslationally to the nascent peptide chain after the removal of 29 amino acids from the COOH-terminus. The contribution of selected COOH-terminal amino acids to the signal for PI-glycan addition was tested by creating a fusion protein with the COOH-terminus of PLAP and a secreted protein and by mutagenesis of specific PLAP COOH-terminal amino acids. The cDNA encoding the COOH-terminus of PLAP was fused in frame to the cDNA for human clotting Factor X and expressed in transfected COS-1 cells. Fusion proteins containing 32 amino acids of the PLAP COOH-terminus were modified by PI-glycan addition. Thus, the signal for PI-glycan modification must reside in these amino acids. Next, the region between the hydrophobic domain and the cleavage site was examined for additional determinants. Mutations of the hydrophilic residues in the spacer region demonstrated that these amino acids do not contribute to the signal for PI-glycan addition. Deletion of amino acids in the spacer region prevented the addition of PI-glycan suggesting that the length of the spacer domain or the amino acids around the cleavage site are important determinants. Finally, we demonstrated that interruption of the hydrophobic domain by a charged residue prevents PI-glycan addition and results in a protein that is secreted into the medium. The finding that a single Leu to Arg substitution in the hydrophobic domain converts a PI-glycan anchored, membrane protein to a secreted protein suggests that an essential signal for the correct sorting of PI-glycan anchored proteins versus secreted proteins resides in the hydrophobic domain. Substitution of a charged amino acid for a hydrophobic amino acid may be a mechanism for producing membrane bound and secreted forms of the same protein.

A number of proteins are anchored in the plasma membrane by a COOH-terminal glycosyl phosphatidylinositol moiety (PI-glycan) (Ferguson and Williams, 1988). cDNA sequences for PI-glycan–anchored proteins predict a typical NH_2-terminal signal peptide that directs the protein to the ER and a 20–30 amino acid COOH-terminal sequence that is absent in the mature PI-glycan–anchored membrane proteins. The PI-glycan attachment occurs in the ER shortly after the peptide chain is synthesized, presumably with the removal of a COOH-terminal peptide (Doering et al., 1990). The modified protein is then transported to the plasma membrane.

The structural determinants that direct the addition of PI-glycan to proteins are only partially understood. Studies of decay-accelerating factor (DAF),[1] Qa-2, and placental alkaline phosphatase (PLAP) suggest that the signals for PI-glycan modification reside in or near the COOH-terminal peptide that is cleaved (Waneck et al., 1988; Berger et al., 1988; Caras et al., 1989). Comparison of the COOH-terminal peptides of PI-glycan linked proteins reveals several common structural features. Each has a hydrophobic domain of varying length, but a consensus sequence for this region is not identifiable. There is a distinct addition site with a preference for amino acids with small side chains (Micanovic et al., 1990; Moran et al., 1991). A spacer region containing hydrophilic and charged amino acids separates the hydrophobic domain and the addition site.

The importance of the hydrophobic domain has been demonstrated for both DAF and PLAP by analyzing the proteins expressed in cells transfected with cDNA containing mutations in the hydrophobic domain (Berger et al., 1988; Caras et al., 1989, 1987). Truncating or deleting the hydrophobic domain prevented the addition of PI-glycan to DAF and PLAP. The hydrophobic domain and 20 additional amino acids from DAF could be fused to the COOH-terminus of glycoprotein D, a herpes virus–encoded protein, or to human growth hormone, creating chimeric proteins that were anchored in the plasma membrane by PI-glycan (Caras et al.,

1. *Abbreviations used in this paper*: CRD, cross-reacting determinant; DAF, decay-accelerating factor; PLAP, placental alkaline phosphatase; VSG, variable surface glycoprotein.

1987). Furthermore, the hydrophobic domain in DAF could be replaced by other hydrophobic sequences without affecting PI-glycan addition (Caras and Weddell, 1989). These studies demonstrate that extensive sequence variation is allowed by the hydrophobic region.

In this study, fusion proteins of human clotting Factor X, a secreted protein, and varying lengths of the COOH-terminus of PLAP were constructed to determine if the entire signal for PI-glycan addition resides in the COOH terminus and to identify the minimum number of amino acids required for PI-glycan addition. After establishing that the COOH-terminal 32 amino acids contain the signal for PI-glycan attachment, various mutations were made in the acceptor amino acid, Asp484, and in selected spacer region amino acids to test their contribution to the PI-glycan modification signal. Finally, a charged amino acid was introduced into the middle of the hydrophobic domain to determine if a charged residue can be accommodated within the hydrophobic domain. The interruption of the hydrophobic domain with a charged amino acid converted PLAP from a membrane-anchored protein to a secreted protein.

Materials and Methods

Construction of Chimeric cDNA and Mutants

The cDNA of human clotting factor X was ligated in frame to synthetic oligonucleotides encoding the 3'-coding region of human PLAP. The Factor X cDNA was in the SmaI site of a pGEM 4z plasmid that had been previously engineered to replace the KpnI site in the polylinker with an XhoI site at the 3' end of the cDNA insert (Sims, H. F. and A. W. Strauss, unpublished data). The Factor X plasmid was digested with XhoI and BstEII to remove 134 bp from the 3' end of the plasmid. A synthetic 50-bp double-stranded oligonucleotide containing bp 1,508 to 1,548 of PLAP and the Factor X BstEII site at the 5' end and an XhoI site downstream from the stop codon (see Fig. 1) was ligated into the plasmid. This oligonucleotide contained a silent, single base mutation that changed bp 1,512 from a G to a T introducing a unique KpnI site. The construct was cleaved at the unique KpnI site and with BstEII and a second double-stranded oligonucleotide spanning bp 1,460 to 1,514 of the PLAP sequence was ligated into the plasmid. The three constructs were then finished by ligating the appropriate synthetic oligonucleotides into the XmaI site at bp 1,471 and the Factor X BstEII site (see Fig. 1).

Mutant sequences were introduced into the cDNA encoding PLAP by cassette mutagenesis. First, the unique KpnI site was introduced into PLAP by ligating the cDNA encoding the PLAP COOH terminus of the fusion protein. The Ala477 plasmid was digested with XbaI and NaeI and the plasmid containing bp 1,441 to the stop codon of PLAP was separated from the Factor X cDNA by agarose gel electrophoresis and isolated by the GeneClean (Bio 101, La Jolla, CA) method following the manufacturer's directions. The plasmid containing the PLAP cDNA was digested with XbaI at the 5' end and StuI at bp 1,160. Another aliquot of the plasmid was digested with StuI and NaeI to give three fragments, a larger one containing pGEM and a portion of the PLAP cDNA, a 688-bp NaeI/StuI fragment, and a 281-bp StuI/NaeI fragment. The 281-bp StuI/NaeI and 1,160-bp XbaI/StuI fragments were isolated from agarose gels by the Gene Clean method. The previously digested plasmid containing the 3' end of the PLAP cDNA, the 281-bp fragment, and the 1,160-bp piece were ligated together to create a plasmid with a 1,550-bp insert containing the entire coding region of PLAP and the unique KpnI site.

The nucleotide sequence of all constructs was verified by dideoxynucleotide sequence analysis (Sanger et al., 1977). All manipulations of DNA were accomplished by standard methods (Sambrook et al., 1989).

Transfections and Analysis of Recombinant Proteins

The cDNA constructs were subcloned into the pSVL expression vector. COS 1 cells were transfected with DEAE-dextran, labeled with [^{35}S]methionine, and the expressed proteins purified with rabbit anti-human PLAP or Factor X antibodies as previously described (Lowe and Strauss, 1990). Phospholipase C digestion, SDS-PAGE and immunoblots were done as before (Lowe and Strauss, 1990). The antibody to the trypanosoma variable surface glycoprotein cross-reacting determinant (anti-CRD) was a gift from A. Gurnett and M. Turner of Merck, Sharp, and Dohme (West Point, PA). For glycosidase treatment, immunopurified proteins were eluted from protein-A Sepharose by boiling for 3 min in 10 μl 0.5% SDS and 0.1 M β-mercaptoethanol. For glycosidase F digestion, 11.0 μl 0.55 M sodium phosphate, pH 8.6, 5.0 μl 7.5% NP-40, 3 μl H_2O, and 300 mU of *N*-glycanase (Genzyme, Boston, MA) were added and the sample incubated under toluene at 37°C for overnight. For endoglycosidase H digestion, 0.25 M sodium phosphate, pH 5.5, and 50 mU endoglycosidase H (Boehringer-Mannheim Biochemicals, Indianapolis, IN) replaced the corresponding components listed above.

Immunofluorescence of Transfected Cells

COS-1 cells were grown to 30–50% confluence on cover slips and transfected with the desired plasmids. After 48–60 h, the medium was aspirated and the cover slips washed three times with PBS. The cells were fixed for 5 min with 4% formaldehyde in cold PBS for surface labeling or with ice-cold acetone for permeabilized cells. The fixative was aspirated and washed away with cold PBS. The cells were then incubated with 1% BSA in 10 mM Tris-HCl, pH 8.0, 0.9% NaCl for 1 h followed by the primary rabbit antibody diluted in 1% BSA, 0.3% gelatin, 25 mM $NaPO_4$, pH 7.5, 0.9% NaCl for 1 h at room temperature. The unbound antibody was washed from the cells with PBS and a fluorescein-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) was added in the same buffer as the primary antibody. After 1 h, the cover slip was washed with PBS and mounted in 50% glycerol in PBS.

Results

Expression of PLAP and Factor X Chimeric Proteins in COS 1 Cells

Initially, two fusion proteins were tested. Ala477 had the COOH-terminal 37 amino acids of PLAP attached to 455 amino acids of Factor X. Thr482 contained the last 32 amino acids of PLAP ligated to Factor X (Fig. 1). Both fusion proteins contained the PI-glycan acceptor site Asp484. Wild type PLAP, Factor X, and the two fusion cDNAs were transfected into COS 1 cells to determine the fate of the expressed proteins. The cells were labeled with [^{35}S]methionine and the immunopurified proteins were analyzed by SDS-PAGE and autoradiography. All four proteins were made in similar quantities by the COS 1 cells and were present in the cell pellet (Fig. 2).

PLAP was detected as two bands in the cell pellet. There was no PLAP present in the medium. In contrast, wild type Factor X was secreted into the medium in significant amounts with only a small amount retained in the cell pellet. The majority of the Factor X was present in the medium as the mature single-chain protein. Factor X is normally processed by a protease into a heavy and light chain and these were also detected in the medium (Leytus et al., 1986). Both Ala477 or Thr482 were detected only in the cell pellet, and like PLAP, migrated as two bands. Neither was secreted by the cells into the medium and there were no detectable processing products. Thus, Ala477 and Thr482 are cell associated and present either on the cell surface or in the cytoplasm.

Cellular Location of the Fusion Proteins

To determine if the fusion proteins were present on the cell surface, transfected cells were analyzed by indirect immunofluorescence. Surface labeling of intact, transfected cells with specific antibodies demonstrated that PLAP and the chimerics were expressed on the cell surface (Fig. 3). In

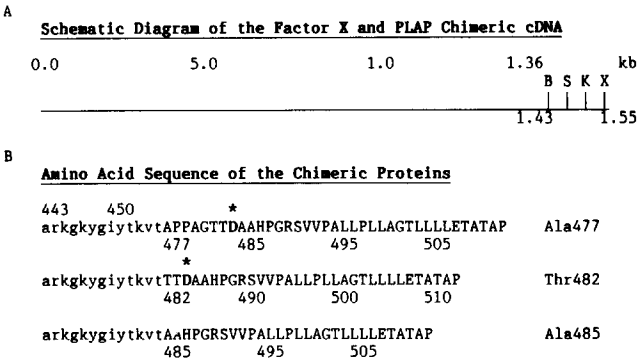

Figure 1. The construction and amino acid sequence of cDNAs encoding Factor X and PLAP fusion protein. *A* shows the schematic diagram of the Factor X/PLAP chimeric cDNA. 1,366 bp out of 1,467 bp in complete Factor X cDNA was fused to the nucleotide sequence encoding the COOH-terminal of PLAP. The unique restriction sites are shown: *B*, BstEII; *S*, SmaI or XmaI; *K*, KpnI; *X*, XhoI. The number of base pairs for the Factor X cDNA are shown above the line. The numbers for the PLAP cDNA are shown below. *B* shows the amino acid sequence for the three constructs using the single letter code. The amino acids derived from Factor X are in lower case. The amino acids from PLAP are in upper case. The numbers above the line are the amino acid position for Factor X. Those numbers below the line are for PLAP. The aspartic acid acceptor is indicated by an asterisk.

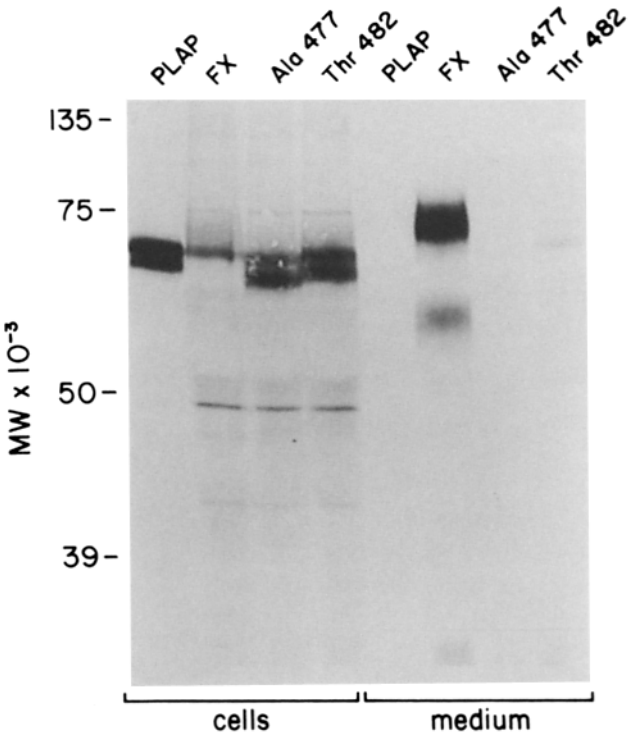

Figure 2. The expression of wild type PLAP, Factor X, Ala477, and Thr482 in COS-1 cells. COS-1 cells were transfected with cDNA, labeled for 2 h with [^{35}S]methionine, and the expressed protein purified from the cells and from the medium by immunoprecipitation with specific antibodies. The cell pellet was lysed in Triton X-100 and deoxycholate-containing buffer and treated with PI-PLC in this experiment. The products were analyzed by SDS-PAGE and autoradiography after transfer to Immobilon P membrane.

contrast, Factor X was not detected on intact cells, but was readily located inside the cells after disruption of the cell membrane with acetone. Although Ala477 and Thr482 are clearly on the cell surface, the mechanism of anchoring the proteins in the cell membrane can not be determined by immunofluorescence.

PI-glycan Addition to the Fusion Proteins

Initial experiments showed that PLAP was readily removed from the COS 1 cells by PI-PLC, but that only a small percentage of Ala477 and Thr482 were removed by PI-PLC (data not shown). Therefore, other approaches were used to demonstrate the presence of the PI-glycan moiety on the fusion proteins. First, the cells were metabolically labeled with [^{3}H]ethanolamine, a component of PI-glycan (Doering et al., 1990). PLAP, Ala477, and Thr482, but not Factor X, labeled with ethanolamine (Fig. 4). Factor X in the medium was also not labeled (data not shown). The presence of ethanolamine in Ala477 and Thr482 suggests that both contain the PI-glycan moiety.

Further evidence for the presence of PI-glycan on the fusion proteins was obtained by testing for binding to an antibody raised against the trypanosoma variable surface glycoprotein (VSG). This antibody (anti-CRD) reacts primarily with the inositol-phosphate that is exposed in PI-glycan after treatment with PI-PLC (Shak et al., 1988). The cell pellets were extracted with buffer containing deoxycholate and Triton X-100 and the cell lysates were treated with PI-PLC. The PI-PLC–treated proteins were purified by immunoprecipitation. [^{35}S]methionine-labeled PLAP, Ala477, and Thr482 migrated as two bands detected by fluorography. Wild type PLAP, Ala477, and Thr482 bound the anti-CRD antibody (Fig. 5). Ala477 and Thr482 that had not been treated with PI-PLC did not bind the anti-CRD antibody. Factor X also did not bind the antibody. In sum, these results—the location on the cell surface, labeling with ethanolamine, and binding to anti-CRD–demonstrate that the fusion proteins contain sufficient information to signal PI-glycan addition and to direct a secreted protein to the plasma membrane.

Importance of the Addition Site in Directing PI-glycan Addition

Both Ala477 and Thr482 contain the PI-glycan addition site, Asp484. To determine if the sequence distal to this site contained the signal for PI-glycan addition a third construct (designated *Ala485* in Fig. 1) was tested. This construct included the 31 amino acids distal to Asp484, but not Asp484. The expressed protein was present in the cell pellet and not in the medium (Fig. 6 *A*, lanes *1* and *2*). There was no detectable release of the protein into the medium by PI-PLC (Fig. 6 *A*, lanes *3* and *5*) and the anti-CRD antibody did not bind to the PI-PLC–treated protein (Fig. 6 *A*, lane *5*). Additionally, the protein was not detected on the cell surface by immunofluorescence on intact cells, but was easily detected in permeabilized cells (Fig. 6 *B*). Thus, the COOH-terminal amino acids adjacent to Asp484 do not, by themselves, allow PI-glycan addition in this fusion protein. A suitable acceptor amino acid, Asp484 for PLAP, must also be present. The absence of a complete signal for PI-glycan addition led to retention inside the cell.

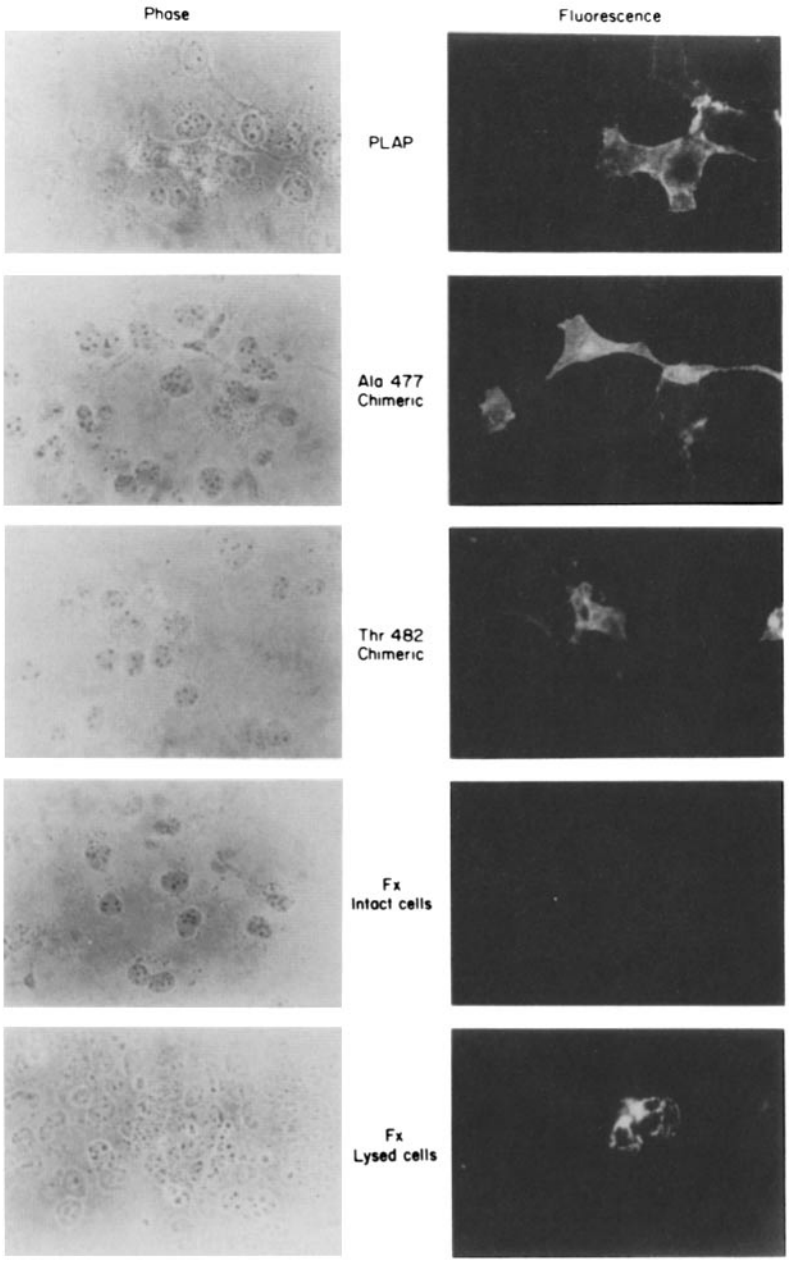

Figure 3. Detection of the expressed proteins by immunofluorescence. Transfected cells were fixed and incubated with specific antibodies to PLAP or Factor X (wild type and chimerics). Binding of the antibody was detected with a fluorescein-conjugated goat anti-rabbit antibody. No fluorescence was detected when the cells transfected with wild type PLAP were incubated with the anti-Factor X antibody or when the Factor X transfected cells were incubated with anti-PLAP antibody.

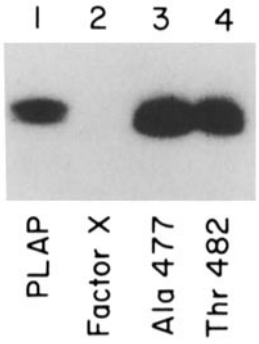

Figure 4. Labeling of the expressed proteins with ethanolamine. The transfected COS-1 cells were labeled with [^{3}H]ethanolamine for 8 h and the expressed proteins in the cell pellet purified with specific antibody. The proteins were analyzed by SDS-PAGE and fluorography. Lane *1* shows wild type PLAP. Lane *2* shows wild type Factor X. Lane *3* contains the Ala477 chimera and lane *4* has the Thr482 chimeric protein.

The Effect of Mutations in the COOH Terminus of PLAP on PI-glycan Addition

Because of the possibility that Factor X may contain signals for secretion that would complicate the analysis of mutations in the PLAP COOH terminus, further analysis of the COOH terminus was done by introducing a variety of mutations into PLAP by cassette mutagenesis. Changes were made in the spacer region, the addition site, and the hydrophobic region. To test the contribution of the hydrophilic residues in the spacer region to the signal for PI-glycan addition, we changed H487, R490, and S491 to a leucine alone or in combination. The effect of increased charge in the spacer domain was tested by changing H487 to an arginine. A deletion mu-

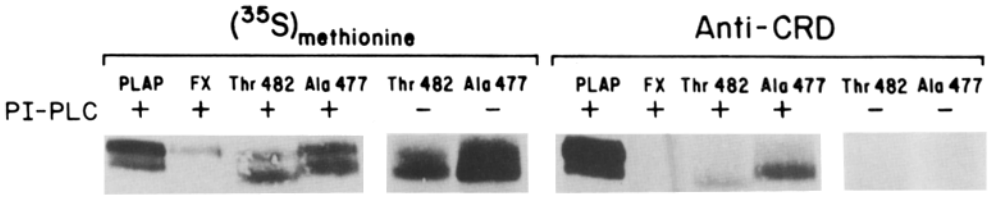

Figure 5. Binding of PLAP, Factor X, Ala477, and Thr482 to the Anti-CRD antibody. The cell pellets from transfected cells were lysed in Triton X-100 and deoxycholate containing detergent and treated for 1 h at 37°C with PI-PLC. The untreated cell pellets were incubated without PI-PLC under identical conditions. The proteins were separated by SDS-PAGE and transferred to Immobilon P membrane. Radiography was done to detect the [35S]methionine-labeled protein and the presence of PI-glycan determined by binding of the anti-CRD antibody. The primary antibody was detected with an alkaline phosphatase conjugated goat anti-rabbit antibody. The PI-plc-treated samples are the same ones shown in Fig. 2.

tant was created in the spacer region to determine the importance of the amino acids adjacent to the PI-glycan acceptor site. The effects of charge, side group size, and polarity at the position of the acceptor amino acid, D484, were examined by mutations at that site. Finally, a charged residue was introduced into the middle of the hydrophobic domain to determine if the hydrophobic stretch could be interrupted by a charged residue as can occur in the membrane domain of a transmembrane protein (Engelman et al., 1986). Each mutant was tested by transfection into COS 1 cells and the presence of PI-glycan determined by susceptibility to PI-PLC cleavage.

As shown by [35S]methionine labeling, all of the mutant proteins were expressed by the transfected cells (Fig. 7 *A*). Two bands were present for some of the mutants and only one band, the faster migrating band, was present for other mutants. Pulse chase experiments with the wild type showed that the faster migrating band was detected initially and that both bands were present at later time points (Fig. 8). After labeling with [35S]methionine for 2 h and treating with glycopeptidase F, PLAP was present as a single band of lower apparent molecular weight than the two untreated bands. Furthermore, endoglycosidase H treatment of PLAP labeled for 10 min produced a single band of the same mobility as the glycopeptidase F-treated PLAP. The differences between the two forms of PLAP is a result of oligosaccharide processing. The lower band contains high-mannose oligosaccharides and is presumably located in the ER.

Only the Leu500 to Arg500 mutant was present in the medium. Thus, the introduction of a single charged amino acid in the middle of the hydrophobic domain resulted in secretion of that protein (Fig. 7 *B*). The secreted protein was

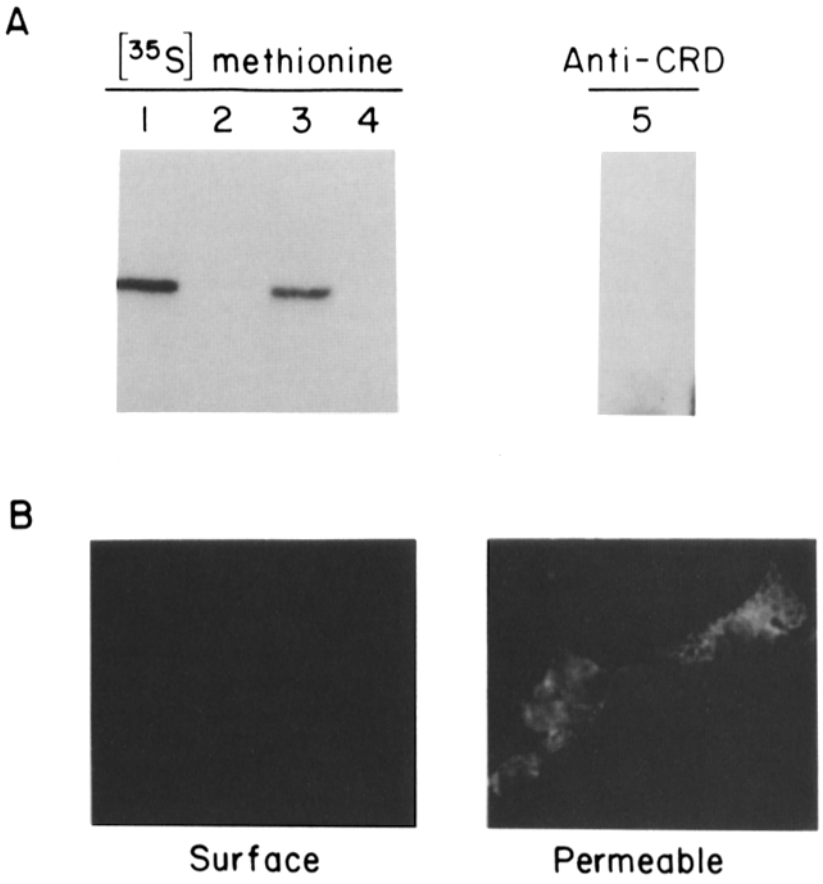

Figure 6. The expression of Ala485 in COS-1 cells and the cellular location of the protein. *A* shows the analysis of the protein purified from [35S]methionine labeled, transfected COS-1 cells by specific antibodies. Lanes *1* and *3* show the cell pellet. Lanes *2* and *4* show the medium. The material in lanes *3* and *4* was from cells treated with PI-PLC. Lane *5* shows the immunoblot of the PI-PLC–treated protein with the anti-CRD antibody. *B* shows the location of the fusion protein in the cells by immunofluorescence. Surface indicates that intact cells were incubated with antibody. Permeable indicates that the cell membrane was disrupted with acetone before incubation with antibody.

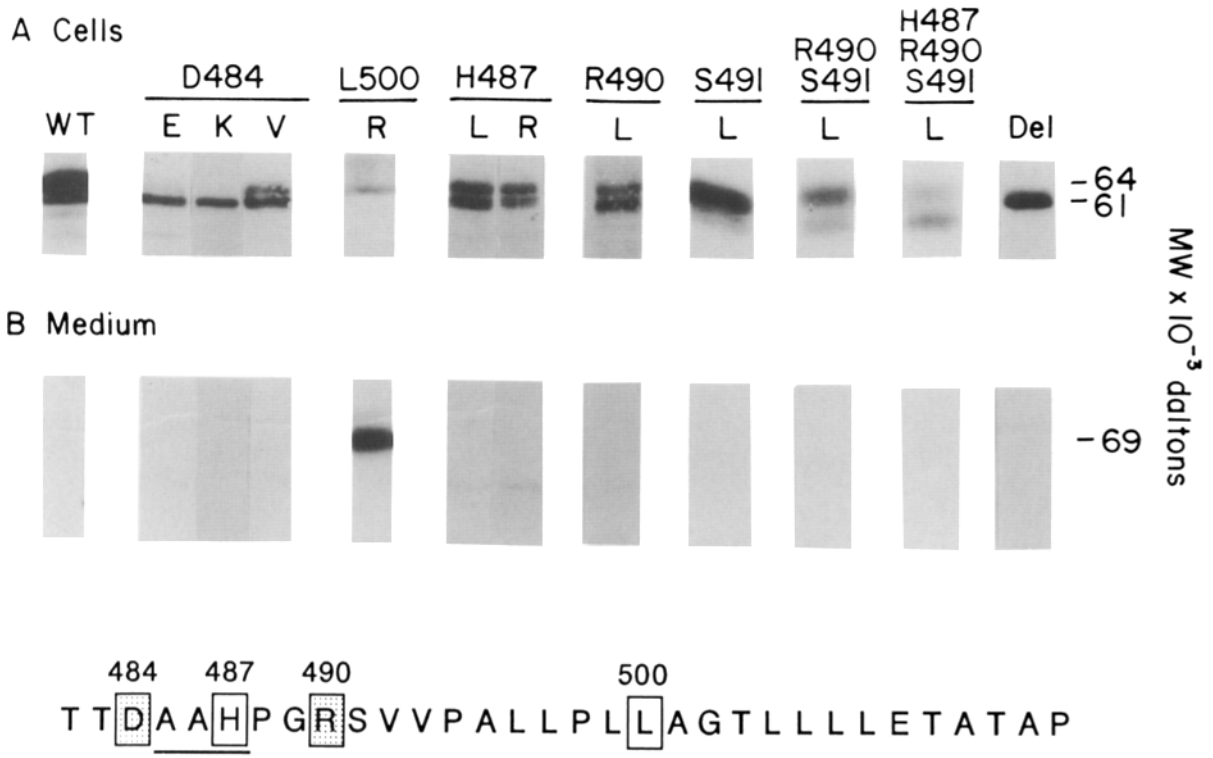

Figure 7. The expression of PLAP mutants in COS-1 cells. Transfected COS-1 cells were labeled with [^{35}S]methionine for 2 h and purified with anti-PLAP antibody. The proteins were analyzed by SDS-PAGE and fluorography. *A* shows the protein in the cell pellet and *B* shows the protein in the growth medium. The wild type amino acid is given above the line and the mutant amino acids below the line. The COOH-terminal amino acids for wild type PLAP are given below. Positions of the changes are marked. The amino acids deleted are underlined.

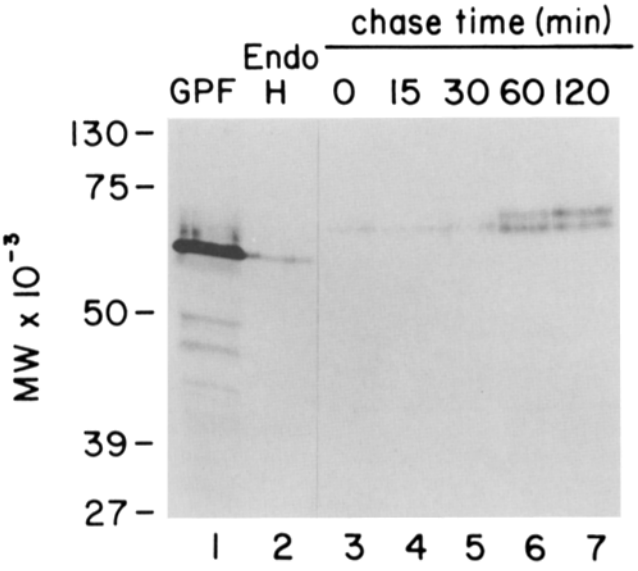

Figure 8. Pulse-chase and endoglycosidase treatment of PLAP. The results of treating PLAP with glycopeptidase F or endoglycosidase H and of a pulse chase are shown here. The transfected cells were labeled with [^{35}S]methionine for 2 h before immunopurification and treatment with glycosidase F. For the pulse-chase and endoglycosidase H experiments the cells were labeled for 10 min. The chase was done with 500× cold methionine in the medium. The chase times are indicated in the figure. Lane *1*, glycosidase F–treated PLAP. Lane *2*, endoglycosidase H–treated PLAP. Lanes 3–7, pulse chase of PLAP.

also larger than the membrane-bound forms, 69 versus 64 kD, suggesting that the secreted form is processed differently from the membrane-bound form. The secreted PLAP had phophatase activity. Approximately 400 mU of AP activity per 100 mm culture dish was present in the medium of the cells transfected with the Arg500 mutant but there was no detectable activity in the medium from cells transfected with wild type or other mutant PLAPs. The results suggest that the Arg500 mutant is not modified by PI-glycan and underscores the importance of an uninterrupted hydrophobic domain for PI-glycan addition.

The presence of PI-glycan was determined by release of the various proteins into the medium after treatment with PI-PLC (Fig. 9). Wild type PLAP was readily released into the medium by PI-PLC. Only one of the three acceptor site mutants, Val484 for Asp484, was cleaved by PI-PLC indicating that valine can support PI-glycan addition (Fig. 9, *D484*). The introduction of the opposite charge, lysine, or of a bulkier but identically charged amino acid, glutamic acid, blocked the addition of PI-glycan.

The only construct with a mutation in the hydrophobic domain was Arg500 for Leu500. Although most of the mutant protein was found in the medium after transfection and was not modified by PI-glycan, there was PLAP associated with the cell pellet, and some of this protein may contain PI-glycan. The cell-associated PLAP R500 was not released by PI-PLC, confirming that PLAP R500 is not a suitable acceptor for PI-glycan (Fig. 9, *L500*).

Proteins that are modified with PI-glycan have hydrophilic

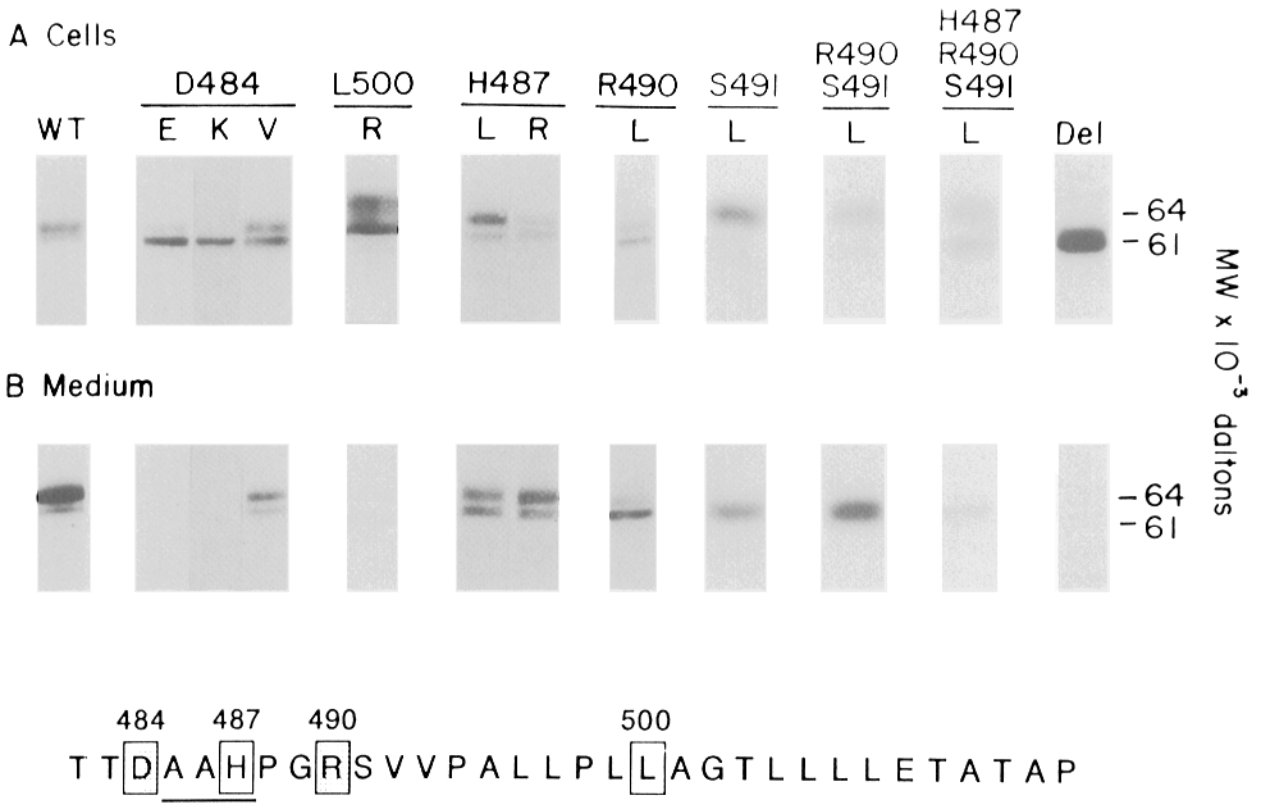

Figure 9. The release of PLAP mutants by PI-PLC. Cells were transfected and labeled with [^{35}S]methionine for 2 h as described in Materials and Methods. The intact cells were treated with PI-PLC for 1 h at 37°C. The PLAP was purified with specific antibodies and separated by SDS-PAGE. *A* shows the protein remaining with the cell pellet. *B* shows the protein released into the medium by PI-PLC. The wild type amino acids are given above the line and the mutants below the line. The COOH-terminal acids of PLAP are given below.

amino acids in the spacer region between the hydrophobic domain and the acceptor site (Ferguson and Williams, 1988). Each of the hydrophilic residues in the PLAP spacer region, His487, Arg490, and Ser491, was replaced with leucine individually and in combinations (Fig. 9). None of the mutations abolished the cleavage by PI-PLC. Additionally, increasing the charge of the spacer domain by changing His487 to a charged residue, arginine, had no effect on PI-PLC cleavage. These results suggest that hydrophilic or charged residues in the region between the addition site and the hydrophobic core are not critical for PI-glycan addition.

In contrast, deletion of three amino acids in the spacer region from Ala485 to His487 blocked release into the medium by PI-PLC. The deletion apparently prevented PI-glycan addition to the mutant protein. Either the amino acids immediately distal to the cleavage site contribute to the

Table I. Sequence of the COOH Terminus of the PLAP Mutants and Their Location by Indirect Immunofluorescence

Mutant	Amino acid sequence	Location
	484 490 500 510	
Wild type	. . .APPAGTTDAAHPGRSVVPALLPLLAGTLLLLETATAP	Surface
Glu484	E	Intracellular
Lys484	K	Intracellular
Val484	V	Surface
Leu487	L	Surface
Arg487	R	Surface
Leu490	L	Surface
Leu491	L	Surface
Leu490,491	LL	Surface
Leu487,490,491	L LL	Surface
Del485-487	---	Intracellular
Arg500	R	Intracellular

The last 37 residues of the COOH terminus of PLAP are shown using the single letter amino acid code. The acceptor site is Asp484. Only the substituted amino acids are given for the mutants. The --- signifies a deletion at that site. The location of the expressed protein in COS-1 cells by indirect immunofluorescence is indicated. Surface means immunofluorescence was detected on the surface of intact cells. Intracellular means that the protein was detected only after disrupting the cell membrane as described in Methods.

specificity of cleavage or the distance between the cleavage site and the hydrophobic domain is important. In this mutant, a proline is placed next to the acceptor aspartic acid. The bulky side chain or helix-breaking properties of proline could prevent cleavage between the aspartic acid and proline.

The location of the expressed proteins was confirmed by indirect immunofluorescence of transfected cells (Table I). The immunofluorescence is consistent with the location of the mutants as determined by PI-PLC susceptibility. As found by others, substitutions that block PI-glycan addition also abolish transport to the cell surface. The proteins accumulate in cytoplasmic compartments (Moran et al., 1991). The Leu500 to Arg500 is the first exception to this general finding.

Discussion

Work with DAF and human growth hormone chimerics suggests that all of the information for PI-glycan addition is contained in the COOH-terminus of DAF (Caras et al., 1989). The location of the signals for PI-glycan addition in PLAP was determined by creating chimerics of human Factor X and various portions of the COOH terminus of PLAP. Factor X is normally a secreted protein and the precursor contains the signals necessary to enter the ER lumen where PI-glycan modification occurs. Furthermore, Factor X does not have a membrane-bound form and should not contain other signals that might direct the protein to the plasma membrane. Thus, the signal directing any of the Factor X-PLAP fusion proteins to the cell surface would be present in the PLAP-derived amino acids. The information for PI-glycan addition was found to reside in the 32 COOH-terminal amino acids containing the addition site and the intact hydrophobic domain. The information present in these amino acids converted a secreted protein, Factor X, into a membrane-bound protein.

The COOH-terminal peptide contains an essential hydrophobic domain and a cleavage/attachment site separated by a spacer region. Mutation of the hydrophilic residues in the spacer domain to hydrophobic residues either alone or in combination did not abolish the addition of PI-glycan. Even though all of the PI-glycan-containing proteins have hydrophilic residues in the spacer region, these results demonstrate that their presence is not required for PI-glycan addition. Alternatively, the distance between the hydrophobic domain and the cleavage site may be crucial.

The spacer domain in PLAP was shortened by deleting three amino acids immediately COOH-terminal to the addition site, leaving only four amino acids in the spacer domain. This mutant remained inside the cell. Caras and colleagues made deletions in DAF that shortened the spacer domain (Caras et al., 1989). Even a mutant missing the normal attachment site was modified by PI-glycan presumably at an alternative site within 8 to 15 residues of the hydrophobic domain. The Ala485 chimeric has a valine 9 residues from the hydrophobic domain and a glycine 14 residues upstream, but neither of these sites is used. Similarly, the DAF and human growth hormone chimeric reported by Moran and coworkers has suitable amino acids 11 and 13 residues NH_3-terminal to the hydrophobic domain and is not modified by PI-glycan (Moran et al., 1991). These results suggest that more than distance from the hydrophobic domain is important in determining the PI-glycan addition site. The addition may also be affected by the residues around the addition site. Thus, the proline next to Asp484 in the PLAP deletion mutant may prevent cleavage or modification. Residues immediately COOH-terminal to the addition site may also be crucial. Lys, Cys, Gly, Ala, Arg, and Thr are located next to the addition site in the proteins where the amino acid sequence is known (Ferguson and Williams, 1988; Moran et al., 1991).

The importance of an uninterrupted COOH-terminal hydrophobic domain is illustrated in our studies by introducing a charged amino acid into the middle of the hydrophobic region. Surprisingly, the Leu500 to Arg500 mutant was not PI-glycan modified or sequestered inside the cell, but was secreted into the medium. Single amino acid changes in Qa-2 and the CD16 IgG Fc receptor converted a PI-glycan–linked protein to a transmembrane protein and truncation of the hydrophobic domain of PLAP produced a secreted protein, but no other single amino acid changes are known to convert a PI-glycan–linked protein to a secreted protein (Waneck et al., 1988; Lanier, et al., 1989; Kurosaki and Ravetch, 1989). This finding raises the possibility that AP found in the serum may be a secreted form of the protein rather than a phospholipase cleavage product of cell surface AP.

Other mutations in PLAP and DAF that prevent PI-glycan addition result in the accumulation of the mutant protein inside the cell, presumably within the ER or Golgi complex (Cara et al., 1989; Micanovic et al., 1990; Lodish, 1988). The mechanism for retention is not clear, but improper folding of the mutant proteins has been proposed as a possibility. Alternatively, changes that affect the cleavage/attachment site may produce proteins that are anchored in the ER membrane and unable to proceed because the PI-glycan moiety is essential for proper targeting. These proteins would also lack the intracytoplasmic domain that may be necessary to route transmembrane proteins to the cell surface. This mechanism predicts that specific sorting of membrane proteins occurs in the ER and that proteins lacking the correct signals are retained in the ER. The leucine to arginine mutation would circumvent the membrane pathway by preventing the nascent peptide chain from anchoring in the ER membrane. Instead, the mutant protein enters the ER lumen and follows the pathway for secreted proteins.

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