Prenylated proteins contain either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid covalently attached to cysteine residues at or near their C terminus. These proteins constitute up to 2% of total cellular protein in eukaryotic cells. The degradation of prenylated proteins raises a metabolic challenge to the cell, because the thioether bond of the modified cysteine is quite stable. We recently identified and isolated an enzyme termed prenylcysteine lyase that cleaves the prenylcysteine to free cysteine and an isoprenoid product (Zhang, L., Tschantz, W. R., and Casey, P. J. (1997) J. Biol. Chem. 272, 23354–23359). To facilitate the molecular characterization of this enzyme, its cloning was undertaken. Overlapping cDNA clones encoding the complete coding sequence of this enzyme were obtained from a human cDNA library. The open reading frame of the gene encoding prenylcysteine lyase is 1515 base pairs and has a nearly ubiquitous expression pattern with a message size of 0 kilobase pairs. Recombinant prenylcysteine lyase was produced in a baculovirus-Sf9 expression system. Analysis of both the recombinant and native enzyme revealed that the enzyme is glycosylated and contains a signal peptide that is cleaved during processing. Additionally, the subcellular localization of this enzyme was determined to be lysosomal. These findings strengthen the notion that prenylcysteine lyase plays an important role in the final step in the degradation of prenylated proteins and will allow further physiological and biochemical characterization of this enzyme.

In eukaryotic organisms, covalent modification by lipids of certain proteins plays an important role in the subcellular localization and biological activities of these proteins (1). One such type of lipid modification is termed prenylation, in which proteins are modified by either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid moiety (2, 3). These prenyl groups are covalently attached to a cysteine residue(s) at or near the C terminus of proteins via a thioether bond (1, 4). A subset of prenylated proteins, termed CAAX proteins, are further modified after the prenylation step. In these proteins, the cysteine residue that is prenylated is initially present as the fourth residue from the C terminus. After prenylation, the three residues after the prenylated cysteine (i.e. the AAX) are proteolytically removed, and the new C-terminal prenylcysteine is modified by methylation (5, 6).

Unlike many other post-translational protein modifications, prenylation is a stable modification of the protein (7). Prenylated proteins have an approximate half-life of 20 h and constitute ~2% of total cellular protein (7, 8). The relatively short half-life of prenyl proteins, along with the stability of the modification, raises a particular metabolic challenge to the cell in the disposal of prenylcysteines resulting from the normal turnover of prenylated proteins. Free prenylcysteines and select analogs have pronounced pharmacological effects on cells, including disruption of cellular signaling that may be attributable to the aberrant localization of specific prenyl proteins (9–11). Another type of post-translational modification, S-acylation, presumably raises a similar metabolic challenge to the cell if the palmitoyl group is not removed from palmitoylated proteins. Recently, a lysosomal enzyme termed palmitoyl protein thioesterase has been shown to remove the palmitoyl group from thiol-containing compounds that appear to result from degradation of palmitoylated proteins (12, 13). Interestingly, defects in this enzyme have been implicated in the neurodegenerative disease infantile neuronal ceroid lipofuscinosis, a lysosomal storage disorder (14).

In an ongoing effort to elucidate the metabolic fate of prenylcysteines in cells, we recently identified an enzyme that catalyzes the degradation of prenylcysteines that we have dubbed prenylcysteine lyase (PCLase; Ref. 15). This enzyme was isolated from bovine brain membranes, a tissue rich in prenylated proteins. PCLase catalyzes the degradation of prenylcysteines to yield free cysteines and a hydrophobic isoprenoid product. The enzyme does this in a manner that is distinct from other enzymes such as carbon sulfur β-lyase, cytochrome P450, and flavin-containing monoxygenases that have been previously shown to act on prenylcysteines (15). In addition, PCLase exhibits a significantly lower $K_m$ and higher specificity for prenylcysteines than either the β-lyase or the monoxygenases.

Another potential route that has been identified for the cellular disposal of prenylcysteines is by transport out of the cell via P-glycoprotein (16, 17), although it seems unlikely that this process is a primary mode of prenylcysteine removal, because P-glycoprotein is not ubiquitously expressed (18, 19). Taken together, the data suggest that PCLase is the major mecha-
nism by which cells dispose of prenylcysteines.

In the present study, the cloning, primary structure, expression pattern, and cellular localization of PCLase are reported. The enzyme was cloned from a human brain cDNA library, and recombinant enzyme was produced in an Sf9 expression system for analysis. Additional studies revealed that PCLase is glycosylated and is localized to the lysosomal compartment in cells. This study provides insights into the role(s) of PCLase and how this enzyme might be involved in the cellular metabolism of prenylated proteins.

**EXPERIMENTAL PROCEDURES**

**Peptide Sequencing—**PCLase was purified from the membrane fraction of bovine brain as described previously (15). Peptide sequencing was performed at both the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University and the Howard Hughes Medical Institute at the University of Texas Southwestern Medical Center. Briefly, 13 μg of purified bovine brain PCLase was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the gel slice containing the protein was excised and subjected to trypsin treatment in situ. Peptides were extracted from the gel, purified by high-performance liquid chromatography (HPLC), and then subjected to Edman degradation reactions. The sequence of six internal peptides was determined. In addition, the N-terminal sequence of the mature protein was determined by sequencing an aliquot of the protein before trypsin digestion.

**Cloning of the Human PCLase cDNA—**Standard cloning techniques were used throughout these procedures (20). Degenerate PCR oligonucleotides were designed using the peptide sequences generated from the Edman degradation reactions. mRNA (bovine brain poly(A)1; CLONTECH) was reverse-transcribed using an Advantage reverse transcription-to-polymerase chain reaction (PCR) kit (CLONTECH) and used as the source of the DNA in a series of degenerate PCR reactions; a specific DNA product of 159 base pairs (bp) was obtained from the primer pair based on the peptide sequences of internal peptides, termed Int 3 and Int 4 (see Table I). After the sequencing of that product, the DNA sequence was extended by using a specific sense primer near the 3' end of the product in conjunction with a degenerate antisense primer to the peptide Int 6; this reaction generated a 684-bp product. The combined sequences were queried against GenBank and Expressed Sequence Tag data bases, and similarity was observed to several partial combined sequences.

**Sequence Tag data bases, and similarity was observed to several partial combined sequences.**

**Construction of the Recombinant Baculovirus—**A recombinant baculovirus transfer vector was constructed using the Bac-to-Bac system (Life Technologies). The entire open reading frame of human PCLase was amplified by PCR using Pfu polymerase with SalI and NotI linkers at the 5' and 3' ends, respectively. The PCR product was then digested and ligated into pFastbac, and the sequence was verified. This vector, termed pFastbac/PCLase, was then used for transposition in DH10Bac E. coli cells. After selection for transposition, the bacmid was isolated and transfected into Sf9 cells using Cellfectin transfection reagent (Life Technologies), and the virus was isolated.

**Expression of PCLase and Isolation of Membranes—**A spinner culture of Sf9 cells in 500 ml of Grace's media supplemented with 10% fetal bovine serum and 1% pluronic was seeded at a density of 0.1 million cells/ml and then infected with either the virus carrying PCLase virus or a control virus. Cells were harvested 52 h after infection, and the cell cultures were disrupted and processed using the 50% Percoll homogenization buffer (20 μl Tris-HCl, pH 8.0, 0.2 μl EDTA, 0.2 μl EGTA, 1 μl dithiothreitol, containing a mix of protease inhibitors; Ref. 22), and the mixture was incubated on ice for 30 min. Cells were disrupted using a glass-Teflon homogenizer, and the suspension was then centrifuged at 40,000 x g for 5 min at 4 °C to remove unbroken cells and nuclei. The postnuclear supernatant was centrifuged at 100,000 x g for 1 h at 4 °C, the resulting supernatant collected, and the membrane fraction was determined. Membranes were aliquoted, flash frozen in liquid nitrogen, and stored at −70 °C until use.

**Synthesis of Radiolabeled Farneyleucysteine—**The synthesis of [35S]-farneyleucysteine was performed as described (15) with the following modifications. The synthesis reaction used [35S]cysteine (NEN Life Science Products; packaged with dithiothreitol as a protectant) at a final specific activity of 0.4 Ci/mmol. A 5-fold excess (relative to the total thiol concentration) of farnesyl bromide (Allrich) was used in the reaction, which proceeded essentially to completion. The [35S]-farneyleucysteine was then purified by HPLC, dried under vacuum, and dissolved in Me2SO for storage at −20 °C.

**PCLase Activity Assays—**To examine the time course of product formation, a bulk reaction containing 7 μg [35S]-farneyleucysteine (−50,000 cpm/time point) in 50 μl Tris-HCl, pH 8.0, at 37 °C was initiated by the addition of either PCLase or control membranes (10 μg/time point). At the times indicated in the appropriate figure legends, 20-μl aliquots were removed, and reactions were terminated by the addition of 10 μl of chromatography development solvent (n-propanol: NH4OH:H2O, 6:3:1). Samples were applied to Whatman LK6D Silica Gel Thin Layer Chromatography plates, and the TLC spots were transferred to Whatman 3MM plates and developed in a solvent system. After development, the plates were sprayed with En'Hance (NEN Life Science Products) and exposed to x-ray film for fluorographic visualization. Both the farnesyleucysteine substrate and cysteine product were scraped from the plates and quantitated by scintillation spectroscopy. For other assays, identical reactions were carried out for 30 min at 37 °C in a final volume of 20 μl with the protein.
concentration noted in the appropriate figure legends. Reactions were terminated and processed as described above.

**Optiprep Density Gradient Fractionation**—The following procedures were all performed at 4 °C unless otherwise noted. Bovine liver (2.5 g) was homogenized in 10 mM Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose, and protease inhibitors (22) using a glass-Teflon homogenizer (10 strokes at 600 rpm). The homogenate was centrifuged at 3,000 × g for 10 min, and the resulting supernatant was subjected to a 12,500 × g centrifugation for 15 min. The membrane pellet from this procedure was resuspended in the same buffer and subjected to an equal volume of 40% Optiprep (Nycomed) in 20 mM Hepes, pH 7.4, containing 2 mM EDTA and 80 mM sucrose. The sample was loaded into a 30-ml centrifuge tube, overlaid with homogenization buffer, and subjected to centrifugation at 100,000 × g for 3 h. Fractions (1 ml) were collected from the bottom of the tube and assayed for catalase (peroxisomal marker), α-mannosidase II (golgi marker), and cytochrome c oxidase (mitochondrial marker) as described (23). Additionally, acid phosphatase activity (lysosomal marker; Ref. 24) and PCLase activities (see above) were determined.

**Immunofluorescence Confocal Microscopy**—HEK293 cells were seeded at a low density on glass coverslips and allowed to grow for 1 day, whereupon the cells were fixed in 4% formaldehyde in phosphate-buffered saline for 10 min and then treated with 10% calf serum in PBS to block nonspecific binding sites. The fixed cells were incubated with PCLase antiserum AP130 and an anti-mouse IgG (Sigma) conjugated to Texas Red (Molecular Probes) for 1 h. The cells were washed three times in IF buffer, and the coverslips were mounted onto a glass slide and sealed. Microscopy was performed using a Zeiss Axiovert 100 confocal microscope. Cells were imaged using a 63× magnification oil immersion lens at 488 nm and 568 nm to excite the Oregon Green and Texas Red conjugates, respectively. Additional markers were used to identify different cellular structures, including lysosomes, peroxisomes, and mitochondria.

**RESULTS**

**cDNA Cloning of PCLase**—Peptide sequences that were identified from N-terminal analysis and tryptic digests of purified PCLase are presented in Table I. To initiate the cloning of PCLase, a PCR approach was taken (see “Experimental Procedures”), which resulted in the generation of a 684-bp PCR product. Several of the peptides identified from the peptide sequencing were found encoded in the 684-bp product, indicating that a partial cDNA encoding PCLase had been obtained. Queries to GenBank revealed similarities to several human expressed sequence tags and also to a region of the noncoding strand of an enzyme termed bile salt-activated lipase (see “Discussion”). Using the sequence of the human PCLase as a guide, primers were designed to several of the expressed sequence tags, and the Gene Trap method was used to screen a human brain cDNA library. Several large (>3-kilobase pair) clones were isolated, but all seemed to lacking

![Table I](image_url)
an initiator methionine, because no product could be produced from the cDNAs in an in vitro transcription-translation reaction (data not shown).

To complete the cloning of PCLase, a technique termed 5’ RACE was performed (see “Experimental Procedures”). The RACE reaction was repeated several times using different 3’ gene-specific primers to ascertain that the true 5’ end was amplified; all products started in the same position. The full PCLase cDNA obtained via ligation of the RACE product into the cDNA obtained from the Gene Trap was 4.2 kilobase pairs in size, in which an open reading frame of 1515 nucleotides was found (Fig. 1). The final cDNA obtained for PCLase contained a relatively short 5’ untranslated region and a large 3’ untranslated region (data not shown). The first methionine residue in this cDNA lies in an optimal Kozak sequence (25), a motif that is often found just upstream of initiation codons.

The deduced protein sequence of PCLase shows no similarity to any known sequence deposited in GenBank. Interestingly, the N-terminal peptide sequence determined for the mature bovine protein began with a glutamic acid (Fig. 1, arrow) and corresponds to residue 29 in the human cDNA coding sequence. This finding suggests the presence of a 28-residue cleavable signal sequence; such a signal peptide was also predicted by sequence analysis also suggested that this enzyme possessed several transmembrane segments. The presence of a signal peptide and putative transmembrane spans provided the first hint that PCLase might reside in an organelle (see below).

Northern Blot Analysis of PCLase Expression—The tissue distribution of PCLase was examined by Northern blot analysis. RNA blots showed a major transcript with an approximate size of 6 kilobase pairs (Fig. 2). PCLase expression appears to be nearly ubiquitous; message was detected in every tissue tested, liver consistently showed the highest level of message. In some tissues, a faint band was also observed at 1 kilobase pair, which may be attributable to an alternative splice site within the gene, mRNA breakdown, or possibly a gene related to PCLase.

Posttranslational Modifications of PCLase—In addition to a predicted signal peptide, several potential N-linked glycosylation sites (residues 196, 323, and 353) were identified in the sequence of PCLase. The presence of the predicted signal peptide suggested that the enzyme would translocate into the endoplasmic reticulum, where it would likely be glycosylated at those accessible sites. As a first test of whether the enzyme was in fact glycosylated, purified bovine enzyme was incubated with two enzymes capable of removing attached sugars, endoglycosidase H and peptide N-glycosidase F. Both glycosidases could indeed digest the native PCLase to faster-migrating species as revealed by SDS-PAGE (Fig. 3A), indicating that PCLase is glycosylated on one or more sites. Digestion of the bovine PCLase was performed in a similar manner to the native protein, an in vitro transcription-translation system was used. This was performed...
in the presence and absence of canine pancreatic microsomes containing the processing machinery to allow full maturation of the newly synthesized protein. The pCMV-Sport/PCLase vector was used in this reaction, and transcription was driven from the SP6 promoter. After translation the samples were split, and half was treated with endoglycosidase H before analysis by SDS-PAGE. Transcription and translation in the absence of added microsomes resulted in formation of a 50-kDa product (Fig. 3B, lane 2), the mobility of which was unaltered by treatment with endoglycosidase H (Fig. 3B, lane 3). Addition of microsomes to the in vitro reaction resulted in the formation of a 64-kDa species (Fig. 3B, lane 4) that comigrated with the purified bovine enzyme. Digestion of this product with endoglycosidase H produced a faster-migrating species than the original translation product (Fig. 3B, lane 5) consistent with the removal of the glycosyl moiety(s) and cleavage of a signal peptide. Taken together these data indicate that mammalian PCLase is processed by removal of a signal peptide and N-glycosylation to produce the mature protein.

Expression of Recombinant Human PCLase—As a final confirmation that the cDNA identified encoded PCLase and to produce a system for future structure-function and other studies, expression of recombinant enzyme was undertaken. A recombinant baculovirus carrying the entire open reading frame of human PCLase was constructed (see “Experimental Procedures”) and used to infect Sf9 cells. Immunoblot analysis of Triton X-100 extracts of membrane fractions derived from PCLase-infected Sf9 cells with a PCLase-specific antibody revealed expression of a protein (Fig. 4, lane 2) that comigrated with the purified bovine protein (Fig. 4, lane 3). Mock-infected Sf9 cell membranes did not produce a protein species that comigrated with the purified enzyme (Fig. 4, lane 1). The presence of a doublet in membranes from the PCLase-infected cells may be a result of incomplete glycosylation of the enzyme or possibly degradation of the enzyme during the processing of the samples.

The enzymatic activity of PCLase in the membrane fraction derived from infected Sf9 cells was examined. Consistent with the immunoblot data, a dramatic (>100-fold) increase in PCLase activity was observed in membranes from cells expressing recombinant protein compared with those infected with baculovirus not carrying the PCLase gene (Fig. 5); very little endogenous activity was detected in the membrane fraction of mock-infected cells. This negligible background activity will facilitate future structure-function analysis of the enzyme expressed in the Sf9 system.

Subcellular Localization of PCLase—Based on the findings that PCLase was glycosylated and possessed a cleavable signal peptide, the subcellular localization of the protein was undertaken. Identification of a specific subcellular location of the enzyme could give insights into the role of PCLase and how it fits into the overall scheme of the metabolism of prenylcysteines. A two-pronged approach was undertaken in an effort to avoid some of the ambiguity commonly seen in these types of experiments. The first approach involved density gradient separation of subcellular components. Bovine liver was homogenized in a sucrose buffer, and the insoluble fraction was isolated and subjected to separation on a 20% Optiprep gradient (see “Experimental Procedures”). Fractions from the gradient were assayed for the presence of mitochondria, lysosomes, peroxisomes, and golgi using the specific marker enzymes cytochrome c oxidase, acid phosphatase, catalase, and α-mannosidase II, respectively (Fig. 6). Analysis of fractions from the Optiprep gradient revealed that PCLase activity comigrated with those of the lysosomal marker in this gradient. Immuno-
blot analysis confirmed that PCLase was present only in those fractions in which activity was detected (data not shown).

The second approach used to determine lysosomal localiza-

tion of PCLase was an immunofluorescence approach that in-
volved examination of colocalization with a known lysosomal
resident protein termed LAMP1 (26). HEK293 cells were used
in this study, because Northern blot analysis indicated PCLase
expression in the kidney (see Fig. 2). Staining of HEK293 with
antibodies to both PCLase and LAMP1 was performed. PCLase
staining revealed a non-nuclear punctate pattern (Fig. 7A),
which was similar to the staining of LAMP1 in the same cells
(Fig. 7B). The overlay of these two panels (Fig. 7C) showed
that there was considerable overlap between LAMP1 and PCLase,
confirming the subcellular fractionation data that PCLase is a
lysosomal enzyme. The lysosomal compartment is the primary
degradative compartment of the cell and is important in the
metabolism of many types of compounds. Lysosomal localiza-
tion of PCLase would suggest that the enzyme does indeed play
a role parallel to that of the aforementioned palmitoyl protein
thioesterase, i.e. the disposal of lipid-modified cysteines in the
cell.

**DISCUSSION**

The present study details the cloning, primary structure,
expression, and subcellular localization of a human prenylcy-
testeine lyase. The cDNA sequence of the cloned enzyme predicts
an open reading frame of 1515 nucleotides encoding a 505-
residue polypeptide. Northern blot analysis showed a nearly
ubiquitous expression pattern with the highest level found in
the liver. Both sequence analysis and experimental data dem-
strate the presence of a cleavable signal peptide in PCLase,
and experiments using both the native bovine enzyme and
recombinant protein revealed that PCLase is a glycosylated
enzyme. The presence of a signal peptide and glycosylation
sites provided the initial clues that PCLase is organellar in
nature, and the results from both subcellular fractionation and
immunolocalization studies revealed that the enzyme resides
in the lysosome.

PCLase is a new and novel enzyme, and sequence analysis
programs applied to the data have not indicated identity with
any known protein or protein class. A potentially interesting
finding is that BLAST analysis of the DNA sequence revealed a region between nucleotides 398 and 1059 of the PCLase cDNA that is identical to a region of noncoding strand in the 5′ untranslated region of the cDNA for an enzyme termed human bile salt-activated lipase (data not shown; Ref. 27). This finding suggests that the genes for PCLase and this lipase contain a region arising from a partial gene duplication.

Several lines of evidence point to the existence of a signal peptide in PCLase. N-terminal sequencing of the purified bovine protein revealed that the first residue aligns with residue 29 of the predicted protein sequence of human PCLase. We expect the mature human protein to begin at the same position, because it has a proline at −4 with respect to the putative cleavage site and an alanine at the −1 site, which approximately follows the −3, −1 rule (28).

The primary sequence of PCLase contains several predicted transmembrane segments. The C-terminal 4 amino acid residues of the open reading frame code for a weak endoplasmic reticulum retention signal, KTEL; the optimal sequence for retention is KDEL (29). This suggests PCLase is not an endoplasmic reticulum resident but is transported to another downstream organelle. Indeed, our data point to a lysosomal location for the mature enzyme.

The physiological role of PCLase can be hypothesized from the data presented here and in the previous work (15). Lysosomal localization for PCLase is completely consistent for a role in the degradation of prenylproteins. The removal of prenylcytstaines is probably critical to prevent a disruption of cellular signaling by these compounds (9–11). Prenylcysteines produced from degradation of prenyl proteins could conceivably be routed to the lysosome from a variety of intracellular locations. When the prenylcytstaines reach the lysosome, they would be digested to free cysteine and an isoprenoid moiety. PCLase does not act on prenylated proteins, because it requires substrates to have a free amino group (15). Because PCLase mRNA expression is highest in the liver, one of the principal organs involved in the detoxification of organic compounds in the body, it is conceivable that PCLase in the liver works in conjunction with P-glycoprotein in other tissues to dispose of prenylcytstaines from tissues that have lower levels of PCLase. For example, the brain, which apparently has a low level of PCLase but is very rich in prenyl proteins, may use this method because both the brain and blood-brain barrier are sites that contain high levels of P-glycoprotein (18). Hence, the prenylcytstaines may be pumped out of these cells, enter the bloodstream, be cleared by the liver, and subsequently be degraded by PCLase for either recycling or disposal of the products of the enzyme.

The cloning of PCLase will facilitate the study of this enzyme in terms of both its mechanistic properties and its cell biology. It will be particularly interesting to determine whether this protein can be implicated in lysosomal storage disorders such as that observed with mutations of palmitoyl protein thioesterase (14).

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