thematic review

Thematic review series: Lipid Posttranslational Modifications

Lysosomal metabolism of lipid-modified proteins

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Abstract **Much is now understood concerning the synthesis of prenylated and palmitoylated proteins, but what is known of their metabolic fate? This review details metabolic pathways for the lysosomal degradation of *S*-fatty acylated and prenylated proteins. Central to these pathways are two lysosomal enzymes, palmitoyl-protein thioesterase (PPT1) and prenylcysteine lyase (PCL). PPT1 is a soluble lipase that cleaves fatty acids from cysteine residues in proteins during lysosomal protein degradation. Notably, deficiency in the enzyme causes a neurodegenerative lysosomal storage disorder, infantile neuronal ceroid lipofuscinosis. PCL is a membrane-associated flavin-containing lysosomal monooxygenase that metabolizes prenylcysteine to prenyl aldehyde through a completely novel mechanism. The eventual metabolic fates of other lipidated proteins (such as glycosylphosphatidylinositol-anchored and N-myristoylated proteins) are poorly understood, suggesting directions for future research.**—Lu, J-Y. and S. L. Hofmann. **Lysosomal metabolism of lipid-modified proteins.** ***J. Lipid Res.*** **2006.** 47: **1352–1357.**

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S-PALMITOYL PROTEINS: LYSOSOMAL PALMITOYL-PROTEIN THIOESTERASE AND THE MOLECULAR BASIS OF INFANTILE NEURONAL CEROID LIPOFUSCINOSIS

Many proteins are modified at cysteine residues by fatty acids (usually palmitate). This modification imparts a local hydrophobic character to the protein and is crucial for diverse biological functions involving membrane-protein or protein-protein interactions, including vesicular transport, signal transduction, and maintenance of cellular architecture (reviewed in 1–3). Notable modified proteins include the transferrin receptor (4), nitric oxide synthase (5), acetylcholinesterase (6), G-protein α subunits, many G-protein-coupled receptors such as the adrenergic, serotonergic, and dopaminergic receptors, and cell-signaling molecules such as Src family protein tyrosine kinases and H- and N-p21Ras proteins (reviewed in 7, 8). Neuronal proteins such as neuronal growth-associated protein-43, synaptosomal protein SNAP-25, and postsynaptic density-95 protein are also known to be palmitoylated. Cycles of palmitoylation and depalmitoylation have been described for some of these proteins, but the relevant enzyme(s) that mediates palmitate turnover in any of these processes has not been isolated or fully characterized (reviewed in 3). A leading candidate deacylating enzyme that would operate in the cytosol is acyl-protein thioesterase (9). Overexpression of acyl-protein thioesterase accelerates the turnover of palmitate bound to G protein α subunits (10).

Palmitoylated proteins must also eventually undergo depalmitoylation in the course of their degradation and disposal in the lysosome. To date, only one enzyme has been described that plays a key role in the lysosomal metabolism of *S*-acylated proteins, a palmitoyl-protein thioesterase (PPT1). PPT1 (EC 3.1.2.22; also known as palmitoyl-protein hydrolase) is a lysosomal enzyme that removes fatty acids from their covalent thioester linkage to cysteine residues in *S*-acylated proteins. Mutations in this enzyme cause the fatal inherited neurodegenerative disorder infantile neuronal ceroid lipofuscinosis (INCL) (11). This disorder is characterized by normal early development to the age of 18 months, followed by cortical and retinal atrophy. Clinical manifestations include motor and cognitive decline, seizures, and blindness, with death in the first decade of life. As in other forms of neuronal ceroid lipofuscinosis (NCL), autofluorescent storage material is readily demonstrated by light microscopy. The infantile form is distinguished from other forms of NCL by the characteristic electron microscopic appearance of the storage bodies, which are

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Abbreviations: FAD, flavin adenine dinucleotide; GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; GROD, granular osmiophilic deposit; INCL, infantile neuronal ceroid lipofuscinosis; MARCKS, myristoylated alanine-rich C kinase substrate; NCL, neuronal ceroid lipofuscinosis; PCL, prenylcysteine lyase; PGAM, peptidylglycine α-amidating monooxygenase; PPT1, palmitoyl-protein thioesterase.

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homogeneously granular and osmiophilic in appearance [granular osmiophilic deposits (GRODs)] (12).

PPT1 was first purified from bovine brain (30,000-fold enrichment) on the basis of its ability to cleave palmitate from a model ^{3}H-palmitoylated substrate, H-Ras (13, 14). A number of other *S*-acylated proteins and peptides are substrates, including palmitoylated Gα proteins (14). Fatty acyl-CoAs are also substrates, allowing the chain length specificity of the enzyme to be determined; the fatty acid chain length optimum is between 14 and 18 carbons (14). No detectable hydrolysis is observed for chain lengths <8 or >22 carbons. The turnover number of the enzyme is low (0.44 per second using palmitoyl-CoA as substrate) but similar to that of other lipases. The K_m for palmitoyl-CoA and for palmitoylcysteine is similar (50 μM) (15). The pH optimum varies with the substrate; it is near neutrality with H-Ras but in the range of 4 to 5 with the artificial substrate 4-methylumbelliferyl-*S*-palmitoyl-β-glucoside (16). The 37 kDa, 306 amino acid enzyme contains motifs characteristic of other thioesterases (a G-X-S-X-G in the N-terminal half of the protein and a G-D-H near the C terminus). The localization of the gene encoding PPT1 to human chromosome 1p32 led to the recognition of its involvement in INCL (11).

The X-ray crystallographic structure of PPT1 (determined with and without bound palmitate) has provided insights into the molecular basis for the phenotypes associated with known PPT1 mutations (17). The enzyme is a globular protein with a classical α/β hydrolase fold typical of lipases. The palmitate rests in a hydrophobic groove down the center of the enzyme; the peptide binding pocket is also easily discernible (**Fig. 1**). The classical hydrolytic catalytic triad consists of serine-115, aspartate-233, and histidine-289. Not surprisingly, mutations that affect residues near the active site and in the hydrophobic core of the enzyme are associated with a severe phenotype, whereas mutations in the binding pocket or at the periphery of the enzyme allow for residual activity and are associated with late-onset disease (**Fig. 2**). Serine is the active site nucleophile, yet like many lipases, the enzyme is resistant to inhibition by the alkylating agent PMSF. This was determined to be attributable to steric hindrance by the phenyl ring at the active site cleft (18). The enzyme is readily inactivated by a serine-reactive alkylating agent (hexadecylsulfonyl fluoride) that mimics the lipid substrate. Many lipases (including PPT2, a lysosomal palmitoyl-CoA hydrolase homologous to PPT1) possess a movable lid domain that regulates interfacial activation of the enzyme. However, PPT1 does not have such a lid; the crystallographic evidence suggests that the palmitate binding surface cleft is stabilized in the permanently open conformation (17).

Most soluble lysosomal enzymes are targeted to lysosomes through a phosphate modification on mannose residues of asparagine-linked oligosaccharides via binding to the mannose 6-phosphate receptor. The binding may occur either within the secretory pathway or from the cell surface, as in the case of exogenously added lysosomal enzymes. The lysosomal targeting of PPT1 occurs through this classical mannose 6-phosphate receptor pathway, at least in periph-

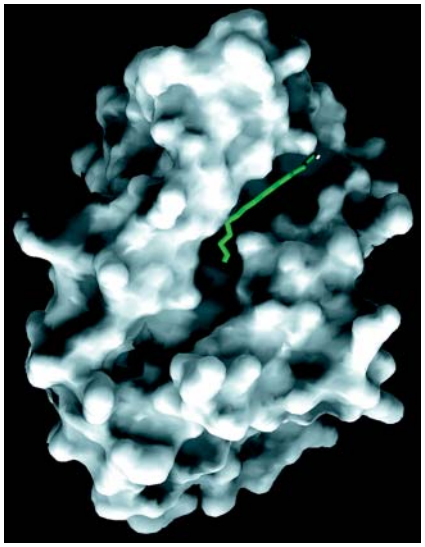

Fig. 1. Molecular surface model of palmitoyl-protein thioesterase (PPT1) with bound palmitate. The palmitate (green) occupies a deep, narrow groove on the hydrophobic face of the protein. The peptide binding pocket is unoccupied. Reproduced from (17). Copyright 2000 National Academy of Sciences, U.S.A.

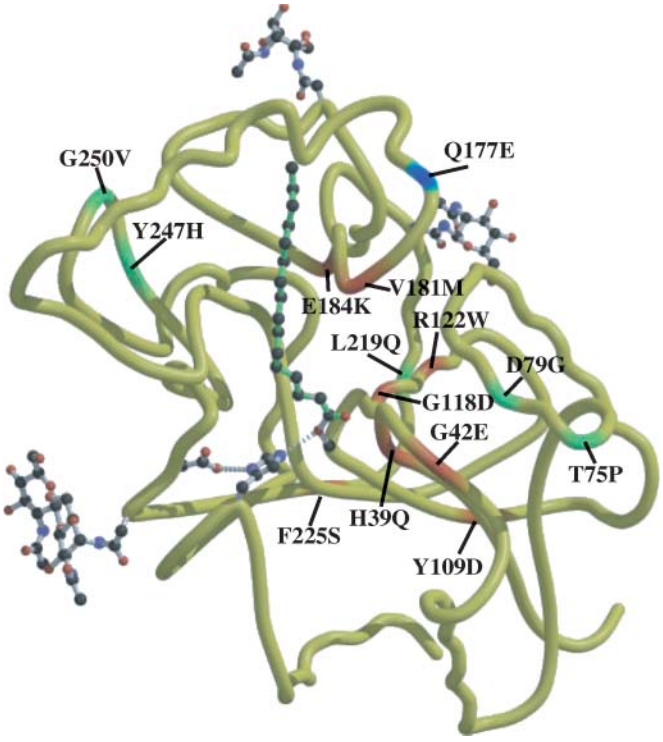

Fig. 2. Mutations in PPT1 causing neuronal ceroid lipofuscinosis (NCL). Sites of clinical NCL mutations in PPT1 are mapped onto the peptide backbone. Infantile-onset mutations are displayed in red, a mutation causing late-infantile NCL symptoms is shown in blue, and juvenile-onset NCL mutations are shown in green. The most severe mutations (green) are found near the active site or within the hydrophobic core of the enzyme and would affect proper folding. Reproduced from (17). Copyright 2000 National Academy of Sciences, U.S.A.

eral tissues (19–21). PPT1 has three asparagine-linked glycosylation sites, at least two of which have been shown to contain the mannose 6-phosphate modification (asparagine-197 and asparagine-232) (22). The role of PPT1 in the lysosomal degradation of acylated proteins has been demonstrated through metabolic labeling studies (23, 24). [^{35}S]cysteine labeling of cells from PPT-deficient patients leads to the accumulation of a number of hydrophobically labeled compounds containing fatty acid and cysteine; their formation is blocked by preincubation of the cells with cycloheximide, indicating that the accumulated material (presumably fatty acylated peptides) is derived from labeled proteins. Furthermore, lysosomotropic agents also block their formation, further implicating PPT1 in the lysosomal metabolism of lipid-modified proteins.

Genetically engineered PPT1 knockout or mutant mice faithfully reproduce many features of the human disease (25, 26). They develop autofluorescent storage material in the brain and other tissues within 6 weeks after birth, and the storage material has the ultrastructural appearance characteristic of INCL (GRODs). The mice also develop myoclonic seizures and spasticity and die by 10 months of age. Electrophysiological studies using cortical neuronal cultures from PPT1 knockout mice exhibited evidence of a reduction in synaptic vesicle number per bouton with time (along with a progressive decrease in synaptic miniature currents) as well as an increase in lysosomal pH, whereas a number of passive and active membrane properties of the neurons are normal (27). The decline in synaptic vesicle number per bouton would appear to represent an early indicator of synapse degeneration and may potentially explain key features of the clinical disease, such as myoclonus and seizures.

The steps leading from enzyme deficiency to eventual neuronal death are only beginning to be delineated. Neuronal cell death (particularly cortical atrophy) is striking in the disorder. At autopsy, brain weight is a fraction of normal and neurons are scarce. Although metabolically labeled lipidated peptides can be found within the storage material, they constitute only a small proportion of its mass. In fact, massive storage is not a feature of the disease, and one can only speculate that the proximal PPT1 substrates are sufficiently toxic to neurons that cell death intervenes before appreciable substrate accumulation can occur. This stands in contrast to "typical" lysosomal storage disorders, in which massive accumulation of substrates of the deficient enzyme is readily demonstrated. Major protein components of the GROD storage bodies are saposins A and D, which are not lipidated proteins but rather cofactors for other lysosomal enzymes (28). Their accumulation is not specific for PPT1 deficiency, although they accumulate to higher levels in INCL than in other lysosomal storage disorders. Abnormal saposin processing, related to a defect in receptor-mediated endocytosis, may underlie saposin accumulation (29). Other potential mechanisms contributing to neuronal death include defects in pH regulation (27, 30), upregulation of inflammatory cytokines (26), and induction of the unfolded protein response with activation of apoptotic pathways (31). Phosphorylation of the endoplasmic reticulum stress-associated translation initiation factor eIF2α, marked upregulation of two well-characterized stress-responsive genes (ATF3 and XBP-1), and activation of the initiator caspase (caspase-12) and effector caspase (caspase-3) have all been demonstrated in the brains of PPT1 knockout mice (31). Increased levels of caspase-9, increased cleavage of caspase-9, and increased reactive oxygen species have also been reported (32).

PRENYLATED PROTEINS: PRENYLCYSTEINE LYASE, A UNIQUE FLAVIN ADENINE DINUCLEOTIDE-DEPENDENT THIOETHER OXIDASE

A detailed understanding of the metabolism of prenylcysteines has been gained through a series of papers from the Casey laboratory beginning in 1997 (33–37). Incubation of ^{35}S-labeled farnesylcysteine or geranylgeranylcysteine with bovine brain extracts was found to generate free [^{35}S]cysteine and an isoprenoid lipid; the free cysteine could be separated from unreacted substrate by butanol extraction. The activity catalyzing this reaction was found in both soluble and membrane fractions and was purified 2,500-fold from bovine brain membranes in a yield of 5–10% by conventional column chromatography (33).

The purified enzyme, dubbed prenylcysteine lyase (PCL) (EC 1.8.3.5; also known as prenylcysteine oxidase), migrates as a single 63 kDa band on SDS-polyacrylamide gels. The K_m for farnesylcysteine and geranylgeranylcysteine is 0.69 and 0.84 μM, respectively. There is a slight preference for farnesylcysteine over geranylgeranylcysteine (V_{max} of 3.9 vs. 1.8 μmol/mg/h). Importantly, the enzyme requires a free amino group for substrate recognition; prenyl peptides and *N*-acetylprenylcysteine are not substrates. Therefore, it seems that prenylated proteins must be completely processed to prenylcysteine before PCL can act.

A human cDNA clone encoding the enzyme was obtained based upon the amino acid sequence of the purified bovine enzyme (34). Northern blot analysis showed an ~6 kb major transcript, which was ubiquitously expressed, with highest expression in liver. The open reading frame is 1,515 nucleotides, encoding a 505 residue polypeptide. An N-terminal 28 amino acid cleavable signal peptide was identified, as well as three asparagine-linked glycosylation sites at residues 196, 323, and 353. Several membrane-spanning segments are suggested by the sequence, and the enzyme expressed in Sf9 cells is tightly membrane-associated. Lysosomal localization was shown by subcellular fractionation of bovine liver and by colocalization of expressed PCL with lysosomal markers in HEK293 cells.

A series of elegant experiments showed PCL to be a novel flavin adenine dinucleotide (FAD)-dependent thioether oxidase (35, 36). The isoprenoid product was shown to be the C-1 aldehyde (farnesal or geranylgeranial). Molecular oxygen was required as a cosubstrate. A noncovalently bound flavin cofactor is used in the reaction, which

is unique in not requiring NADH or NADPH. A stoichiometric amount of hydrogen peroxide is generated during the course of the reaction. The simplest mechanism that accounts for all three products of the reaction is shown in **Fig. 3**. The proposed mechanism (oxidative cleavage of a thioether bond without net oxidation at sulfur) appears to be unique and unprecedented.

Digits et al. (36) used product and dead-end inhibitors to probe the kinetic mechanism of PCL. The simplest mechanism, as currently understood, is a six step reaction that proceeds through a sequential mechanism. The reaction is initiated by stereospecific transfer of the pro-*S* hydride at C-1 of farnesylcysteine to FAD (Fig. 3). This produces a reduced flavin and probably a short-lived carbocation intermediate. Attack of a water molecule on the carbocation results in the formation of a hemithioacetal intermediate, which then forms the isoprenoid aldehyde, with C-S cleavage to produce cysteine. Cysteine production is one of the (perhaps the major) rate-limiting steps of the reaction. The hydride of the reduced flavin is transferred to molecular oxygen to form hydrogen peroxide, regenerating the oxidized flavin for the next catalytic cycle.

Knockout mice with targeted disruption of the PCL gene demonstrated no significant physiological effects (37), although PCL activity in knockout cells was substantially reduced. Farnesylcysteine and geranylgeranylcysteine accumulated to high levels in knockout tissues. Farnesylcysteine levels were 50-fold increased in liver and 40-fold increased in brain tissue; the corresponding tissue levels of geranylgeranylcysteine were 10- and 30-fold higher, respectively. In contrast to tissue levels, blood levels of these compounds were not distinguishable from those of wild-type controls. Lipofuscin accumulation was not demonstrated; life span and reproduction were normal. Although many mouse models do faithfully recapitulate human lysosomal storage diseases, the negative results in PCL-deficient mice do not rule out the possibility of a human phenotype given the differences in longevity between species and the cumulative nature of storage disorders. A paralog that is 43% identical to PCL over 473 amino acids is present on human chromosome 5q33.1. No published information concerning this hypothetical protein (MGC3265) is available. It is possible that residual PCL activity arises from this or another related gene. At this writing, no putative disorders have been reported in the human chromosomal location for PCL at 2p13.3.

GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED AND *N*-MYRISTOYLATED PROTEINS

There is ample information on the synthesis of glycosylphosphatidylinositol (GPI)-linked proteins, including the identity of >20 different proteins involved in GPI anchor synthesis and 6 proteins involved in the transamidase complex that processes the nascent protein and catalyzes transfer of the anchor to protein (reviewed in 38). In contrast, the metabolism of GPI-anchored linked proteins has been relatively unexplored. Proteins may be released from their GPI anchors through the action of glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) or possibly a phosphatidylinositol-specific phospholipase C (reviewed in 39). GPI-PLD activity is abundant in serum, cerebrospinal fluid (40–42), and synovial fluid and is secreted by activated neutrophils (43). The activity has also been described as enriched in lysosomal fractions of bovine liver (44). GPI-PLD is taken up into cells from culture medium and proteolyzed to smaller fragments, a process that is inhibited by the lysosomotropic agent chloroquine (45), which raises the possibility of lysosomal

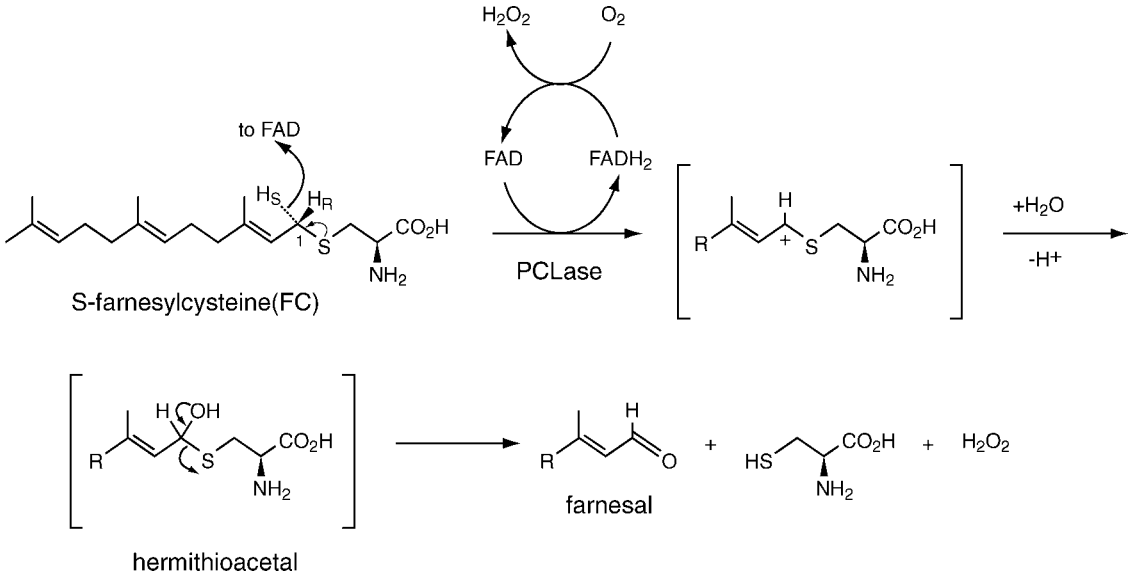

Fig. 3. Proposed mechanism of prenylcysteine lyase. Farnesyl cysteine is oxidized by the flavin to a thiocarbenium ion, which reacts with water to form a hemithioacetal intermediate. Collapse of the hemithioacetal generates hydrogen peroxide, farnesal, and cysteine. The reduced flavin is regenerated to its oxidized form by molecular oxygen. FAD, flavin adenine dinucleotide; PCL, prenylcysteine lyase. Reproduced from (36) with permission. Copyright 2002 American Society for Biochemistry and Molecular Biology.

targeting. Metabolism of GPI anchor intermediates, a function also performed by GPI-PLD, has been shown to be sensitive to chloroquine and ammonium chloride, maneuvers that increase lysosomal pH (46). However, overexpressed GPI-PLD was predominantly localized to the endoplasmic reticulum and lipid rafts (although the lysosomal fraction was not analyzed). Further work is needed to resolve the question of the intracellular metabolism of GPI anchors and GPI-modified proteins and whether a lysosomal GPI-PLD is involved.

N-Myristoylation is generally considered to be a stable, irreversible cotranslational event. Demyristoylation of proteins is a topic that has been little studied. The only available published information concerns one particular myristoylated protein, MARCKS (for myristoylated alanine-rich C kinase substrate). MARCKS is a major cellular substrate of protein kinase C involved in neuronal secretion, cell migration and adhesion, regulation of the actin cytoskeleton, and lipid second messenger signaling (47). Demyristoylation of MARCKS (and the MARCKS-related protein) by a soluble fraction of brain synaptosomes has been reported (48, 49). Production of the full-length but demyristoylated form of MARCKS was confirmed by electrospray mass spectrometry and amino acid sequencing. This confirmation was important because removal of the N-terminal myristate can also occur via proteolysis between lysine-6 and threonine-7 (at least in macrophages) (50). Furthermore, MARCKS demyristoylated by incubation with brain extract can be remyristoylated in vitro. The demyristoylation reaction requires ATP (which was proposed to be necessary to promote a permissive structural change within the substrate, rather than as a cofactor for the enzyme) and is inhibited by the interaction of MARCKS with calmodulin. It is unknown whether the activity resides in the cytosol or in a soluble lysosomal fraction. It is possible that the demyristoylation is a function of *N*-myristoyltransferase operating in reverse, as myristoylation of peptides by *N*-myristoyltransferase is a reversible reaction and can be driven toward demyristoylation in the absence of CoA (51).

Therefore, the existence of a lysosomal activity that mediates the demyristoylation of proteins is only speculative at present. A bacterial *N*-myristoyl-cleaving enzyme has been described (52). Another potential pathway for the metabolism of *N*-myristoylglycine derived from *N*-myristoylated proteins would be conversion to the corresponding amide (myristamide) through the action of peptidylglycine α-amidating monooxygenase (PGAM) (53). The resulting myristamide would then be a substrate for the action of fatty acid amide hydrolase, a membrane-bound enzyme (54). PGAM is present in extracellular fluids, such as serum and cerebrospinal fluid, as are most lysosomal enzymes. Whether PGAM is present in lysosomes or participates in *N*-myristoylglycine metabolism is an open question.

CONCLUSIONS

The lysosomal degradation of lipid-modified proteins requires two key enzymes: PPT1 and PCL. These enzymes are crucial for the metabolism of *S*-fatty acylated proteins and prenylated proteins, respectively. They differ in several important respects. PPT1 is a globular thioesterase that structurally resembles lipases; it catalyzes the simple hydrolysis of palmitoylcysteine and palmitoylated peptides, and it is targeted to lysosomes through the classical mannose 6-phosphate receptor pathway. Deficiency of PPT1 leads to a severe neurodegenerative disorder (INCL) in humans and mice. PCL is a FAD-dependent thioether monooxygenase that metabolizes prenylcysteine but not prenylated peptides, and it is an intrinsic lysosomal membrane protein. PCL deficiency in mice produces no known phenotype. Much remains to be learned concerning the sequence of events that lead from PPT1 deficiency to neuronal cell death, why neurons are affected while other tissues are spared, and what redundant or compensatory mechanisms prevent neurodegeneration in PCL deficiency. Finally, details of the eventual metabolic fate of myristoylated and GPI-anchored proteins are lacking and may provide topics for future research.

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