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Proteomics Clin Appl. Author manuscript; available in PMC 2015 June 01.

Published in final edited form as:
Proteomics Clin Appl. 2014 June ; 8(0): 327–337. doi:10.1002/prca.201300090.

Carboxypeptidases in disease: Insights from peptidomic studies

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Abstract

Carboxypeptidases (CPs) perform many diverse physiological functions by removing C-terminal amino acids from proteins and peptides. Some CPs function in the degradation of proteins in the digestive tract while other enzymes play biosynthetic roles in the formation of neuropeptides and peptide hormones. Another set of CPs modify tubulin by removing amino acids from the C-terminus and from polyglutamyl side chains, thereby altering the properties of microtubules. This review focuses on three CPs: carboxypeptidase E, carboxypeptidase A6, and cytosolic carboxypeptidase 1. Naturally-occurring mutations in all three of these enzymes are associated with disease phenotypes, ranging from obesity to epilepsy to neurodegeneration. Peptidomics is a useful tool to investigate the relationship between these mutations and alterations in peptide levels. This technique has also been used to define the function and characteristics of CPs. Results from peptidomics studies have helped to elucidate the function of CPs and clarify the biological underpinnings of pathologies by identifying peptides altered in disease states. This review describes the use of peptidomic techniques to gain insights into the normal function of CPs and the molecular defects caused by mutations in the enzymes.

Introduction

Most, if not all proteins undergo post-translational modifications that affect the properties of the protein. Well-known modifications include phosphorylation, glycosylation, and proteolysis. The latter group includes over 500 known proteases and peptidases [1]. While commonly thought of as playing a degradative role in the cell, proteases and peptidases can also activate or otherwise modulate the activity of proteins and peptides. Proteases and peptidases are divided into two broad categories based on location of cleavage site within the substrate. Endoproteases/endopeptidases cleave peptide bonds located anywhere in the protein, whereas exoproteases/exopeptidases require an N- or C-terminus near the cleavage site. Aminopeptidases cleave proteins and peptides from the N-terminus, often one or two residues at a time depending on the enzyme. In contrast, carboxypeptidases (CPs) cleave proteins and peptides from the C-terminus, usually one residue at a time. Release of C-

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Conflict of interest statement
The authors have declared no conflict of interest.

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terminal amino acids is a widespread process that plays a role in degradation, processing, and modulation of proteins and peptides.

The largest family of enzymes responsible for cleavage of C-terminal residues is the M14 family of metallocarboxypeptidases [reviewed in 2]. In most mammals, there are 25 distinct genes for M14 family proteins, although not all are known to be active as peptidases. These 25 gene products are divided into four subfamilies based on amino acid sequence homology and domain structure (Fig. 1). The A/B subfamily contains 9 members, including the well-known digestive enzymes CPA1 and CPB1 that cleave C-terminal aromatic/aliphatic amino acids and basic amino acids, respectively. Except for CPO [3], all members of the A/B subfamily are transcribed with an inactivating prodomain which aids in folding and prevents these enzymes from being active until they are cleaved by an endopeptidase. CPO is also the only enzyme in this subfamily that does not have an A-like or B-like substrate specificity, and instead cleaves C-terminal glutamates from peptides [3]. The N/E subfamily consists of 8 proteins, although only 5 have been shown to be enzymatically active peptidases [4]; the other three members of this subfamily lack one or more active site residues that are generally required for catalytic activity [5–7]. Members of the N/E subfamily do not have an inactivating prodomain, and instead contain a C-terminal transthyretin- like domain that is thought to be involved in protein folding. A third subfamily of metallocarboxypeptidases is the cytosolic carboxypeptidases (CCPs). The six members of this subfamily are predominantly localized to the cytosol and nucleus [8–10], and some have been found to modify tubulin [11–13]. Like the A/B subfamily, the CCPs contain a beta sheet-rich domain immediately N-terminal to the metallocarboxypeptidase domain, although this upstream domain does not need to be removed to generate the active form of the enzyme. The fourth subfamily contains two members, both aminoacylases: aspartoacylase and aminoacylase-3. Originally these enzymes were not thought to be related to the M14 family of metallocarboxypeptidases but when their crystal structures were analyzed, it was noted that they fold into the same general structure as other members of the family and likely represent a fourth subgroup of the M14 family [14,15]. Most of the active site groups are conserved between the aminoacylase subfamily and the other three subfamilies, supporting the proposal that these enzymes are in the M14 family.

The 25 metallocarboxypeptidases in the M14 family have different biological roles due to their varying substrate specificities and/or distributions (tissue, cellular, and subcellular). For example, of the nine members of the A/B subfamily, six have CPA-like activity and cleave aromatic and aliphatic residues. However, only two of these (CPA1, CPA2) are located in the acinar cells of the pancreas where their secretion allows them entry to the digestive tract. The substrate specificities of these two enzymes are different; CPA1 prefers smaller hydrophobic residues while CPA2 prefers residues with bulkier side chains such as tryptophan [16]. Of the other four CPA-like enzymes, their tissue, cellular, and subcellular distributions are completely different from each other as well as from CPA1 and CPA2. One of these (CPA3) is restricted to mast cells [17], and the others show limited expression in various adult tissues and during development. Even though CPA4 and CPA6 are expressed in some of the same tissues, their subcellular distributions are different; CPA6 is bound to the extracellular matrix after secretion [18] whereas CPA4 remains soluble after secretion [19], thus giving the two enzymes access to different substrates. Additionally, detailed

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analysis of substrate specificities of CPA3, CPA4 and CPA6 have revealed differences in which residues they prefer in the P1 and P1′ positions [19–21], further differentiating them from one another.

Various diseases have been associated with genetic defects in carboxypeptidases. In mice, spontaneous mutations in the *Cpe* gene cause a syndrome characterized by obesity, anxiety, depression, and infertility [22,23], while spontaneous mutations in the gene encoding CCP1 cause degeneration of a subset of cells in brain, eye, and testis [24,25]. In humans, mutations in the gene encoding CPA6 have been identified in human populations with epilepsy and febrile seizures [26,27]. Studies on these three enzymes are described in more detail in this review. In addition to these enzymes, other carboxypeptidases in the M14 family have been found to produce phenotypes when the enzyme was mutated or deleted. CPD mutations in *Drosophila* have been found to be lethal at the pupal stage [28–30]. CPB2 (also known as plasma CPB, CPR, CPU and TAFI), has been linked to blood clot stability [31]. Mice with deletions of the gene encoding CPB2 demonstrate enhanced fibrinolysis [32], reduced wound healing [33,34], increased inflammatory response to lipopolysaccharide [35], and enhanced aortic aneurysm formation [36]. CPB2 has also been found to decrease inflammatory responses in autoimmune arthritis [37]. Disruption of the gene encoding the active subunit of carboxypeptidase N (*Cpn1*) in mice has been shown to increase susceptibility to C5a anaphylatoxin-mediated shock [38]. Although AEBP1 is an inactive member of the M14 family, mice deficient in this protein display gastroschisis [39] and lactation defects [40], as well as normalization of atherosclerosis induced by some genetic backgrounds [41]. In humans, mutations in aspartoacylase cause Canavan disease [42,43]. Mutations in the genes encoding other M14 family members are likely to cause phenotypes but have not been reported in the literature.

In this review, we focus on three metallocarboxypeptidases (CPE, CPA6, and CCP1) and their role in disease. Importantly, we describe how peptidomics techniques (such as those described in Fig. 2) have helped define the specific role of the enzyme and contribute to a better understanding of the disease process caused by defects in the enzyme.

CPE

CPE was discovered in the secretory vesicles of bovine adrenal medulla and was originally named enkephalin convertase, reflecting its ability to produce the mature form of enkephalin from its precursor [44]. Subsequently, it was found that CPE had a broad neuroendocrine distribution and was present in many cells that did not produce enkephalin, leading to the prediction that this enzyme played a general role in the production of neuropeptides and peptide hormones [45]. The substrate specificity of CPE was consistent with a broad role in the processing of any peptide containing a C-terminal Lys or Arg residue [45].

For many years, CPE was incorrectly thought to be the only CP involved with the production of neuropeptides and peptide hormones. In 1995 it was discovered that mice lacking CPE activity due to a point mutation in the *Cpe* gene (named the *fat* mutation) produce low levels of neuropeptides and peptide hormones [23]. This led to the hypothesis that another CP participated in the production of neuropeptides and peptide hormones;

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subsequent studies identified CPD, a CPE-like enzyme that functions in the secretory pathway [46]. CPD primarily functions in the trans Golgi network [47] while CPE functions later in the secretory pathway within the maturing secretory vesicles. The absence of CPE activity results in abnormally low levels of nearly all neuropeptides and peptide hormones that normally require a CP in their production [23,48]. These findings suggest that CPE plays the predominant role in neuropeptide production, with CPD playing a minor role in the formation of most neuropeptides.

Mice lacking CPE activity due to either a point mutation (i.e. $Cpe^{fat/fat}$ mice) or targeted disruption in the *Cpe* gene (CPE knockout mice) show a number of abnormal behaviors or physiological changes, including obesity, infertility, anxiety, depression, memory deficits, and neurodegeneration of hippocampal neurons [22,49–52]. Several studies have looked for mutations of *CPE* in human populations [53,54]. Although several variants of the *CPE* gene have been found, most do not appear to contribute to any disease phenotype, possibly due to the difficulty in finding recessive mutations in human populations. In the animal models, one copy of active CPE is sufficient to produce a normal phenotype, with no changes in levels of neuropeptides detected in mice heterozygous for the mutation ($Cpe^{fat/+}$ mice), as compared to wild-type littermates. Only one human study reported a correlation of a *CPE* mutation with disease; the onset of diabetes in a population that was prone to type 2 diabetes mellitus [54]. However, the *CPE* variant was found in roughly equal frequency in the population who developed diabetes versus the total population, suggesting that the variant does not cause diabetes in the general population. No humans were observed with both *CPE* alleles mutated, although the population size of the study was too small to reliably detect such individuals if they exist. The observed *CPE* variant is not very common and doesn't account for vast majority of human obesity.

Peptidomics has been used to study $Cpe^{fat/fat}$ mice in two different approaches. The first approach used affinity chromatography on immobilized anhydrotrypsin agarose to purify CPE substrates from $Cpe^{fat/fat}$ mouse brain regions [55]. In the absence of CPE activity there was a dramatic increase in levels of neuropeptide processing intermediates containing C-terminal Lys and/or Arg residues. These intermediates bound to anhydrotrypsin agarose and could be eluted by a change in pH. In addition to neuropeptide processing intermediates, the affinity resin also bound endogenous peptides that contain C-terminal basic residues. By comparing the relative signal strength of the peptides detected in $Cpe^{fat/fat}$ versus wild-type mice, those peptides that were greatly elevated in the mutant mice were assumed to be CPE substrates and these were subjected to MS/MS analysis and their amino acid sequences determined. This analysis led to the identification of several novel neuropeptides, including peptides named SAAS, GAV, PEN, and LEN [56]. All of these peptides are derived from the same precursor, named proSAAS. Differential processing of proSAAS gives rise to big and little forms of each peptide [57]. ProSAAS-derived peptides such as PEN and Big LEN have been implicated in obesity/body weight regulation [58–60]. In addition to the novel proSAAS-derived peptides, a number of known peptides were identified from the MS analysis of peptides purified from $Cpe^{fat/fat}$ mouse brain, including a number that were also implicated in feeding/body weight regulation such as neuropeptide Y, melanocyte stimulating hormone, and others [55].

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A different peptidomics approach was used to compare levels of peptides in *Cpe*$^{fat/fat}$ mouse brain extracts with the levels in extracts of wild-type mouse brain [48,61–63]. In this approach, the peptides were not affinity purified prior to analysis so that the mature forms could be detected along with the Lys/Arg-containing precursors. Peptides extracted from the two groups of mice were labeled with isotopic tags, combined, and analyzed by LC/MS, in a scheme shown in Figure 2C. This analysis revealed CPE substrates as well as products: the substrates were elevated in *Cpe*$^{fat/fat}$ mouse extracts while the products were lower in these extracts, relative to levels in wild-type mice (similar to the strategy described in Fig. 2C, F). As expected from the earlier analysis of affinity purified peptides, the peptides found to accumulate in the *Cpe*$^{fat/fat}$ mouse brain included most neuropeptide processing intermediates. This finding is consistent with the major role of CPE in the formation of most neuropeptides. Peptides that were not greatly affected by the absence of CPE activity generally represented the C-terminus of their precursors; these peptides do not require CPE for their production and instead need only an N-terminal cleavage by an endopeptidase.

The question of why the *Cpe*$^{fat/fat}$ mice are overweight can be addressed by examining the changes in levels of various peptides detected with the quantitative peptidomics technique [64]. Peptides are known to be involved in both sides of the energy balance equation; some peptides are orexigenic and lead to an increase in feeding and body weight while others are anorexigenic and lead to a decrease in feeding and body weight. In the *Cpe*$^{fat/fat}$ mouse, levels of anorexigenic peptides are generally lower than in wild-type littermates. While some orexigenic peptides are also present at lower levels in the *Cpe*$^{fat/fat}$ mice than in wild-type littermates, several orexigenic peptides do not require CPE for their production and are therefore unaltered by the absence of CPE. For example, the proSAAS-derived peptide named Big LEN represents the C-terminus of its precursor and therefore doesn’t require CPE for production of the mature form of the peptide. Similarly, beta-endorphin 1–31, melanin concentrating hormone, and agouti gene-related peptide are also the C-terminal regions of their respective precursors and therefore do not require CPE. All of these peptides have been reported to be orexigenic [58,65–67]. Therefore, even though some orexigenic peptides (such as neuropeptide Y) are decreased in the *Cpe*$^{fat/fat}$ mouse brain, other orexigenic peptides are present at normal levels. In contrast, the vast majority of anorexigenic peptides are greatly reduced by the absence of CPE activity, and so the net change is an increase in the balance between orexigenic versus anorexigenic peptides and as a result the mice eat more and accumulate body fat.

CPA6

CPA6 was discovered in an amino acid homology search of the human genome aimed at uncovering novel carboxypeptidase genes [68]. The gene was discovered on chromosome 8, and was similar to a cDNA from human hematopoietic stem cells previously deposited in GenBank. The cDNA, however, was lacking exons 9–11, which contain critical residues for catalytic function. Subsequent analysis of the region revealed that the missing exons were about 50 kilobases downstream of the end of exon 8 and confirmed the existence of a transcript with the missing 3′ exons [68]. Bioinformatic analysis revealed that the enzyme has the key features of an active carboxypeptidase, including the residues required to coordinate a central zinc ion, and residues directly involved in catalysis. In addition, it

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contains a methionine in the position conferring substrate specificity, which was predicted to give the enzyme a preference for hydrophobic residues, leading to its classification as a member of the A-like subfamily of carboxypeptidases [68].

CPA6 tissue distribution was first studied in the mouse. *Cpa6* mRNA expression was detected in the olfactory bulb, cingulate cortex, lateral septum, pontine nucleus, inferior olivary complex and several other brain regions[69]. In adult mice, *Cpa6* mRNA is most highly expressed in olfactory bulb, where it is present in the mitral and granule cell layers. Outside the CNS, *Cpa6* mRNA is expressed in several tissues in the developing mouse such as the bone marrow, skin, and musculature surrounding the stomach [69]. Further research must be conducted to confirm exactly which cells express *Cpa6*. For example it is not currently known whether neurons and/or glia express *Cpa6* in the murine CNS. In humans, *CPA6* mRNA has been detected in the hippocampus and raphe nuclei [26]. Because CPA6 orthologs in mouse and human have an apparently complex and regulated expression pattern throughout spatial and temporal dimensions, it is difficult to conclude what its function may be from tissue distribution alone.

Cellular distribution, while informative, is complicated by another characteristic of CPA6. Studies using HEK293T cells showed that although the majority of CPA6 was bound to the extracellular matrix, some enzyme was secreted into the cell culture medium [18]. As a secreted enzyme, it is possible that it could act in signaling pathways involving cells distant from those that produce it. CPA6 is transcribed with a prodomain, which is cleaved by furin or a furin-like enzyme upon secretion to form the active enzyme. CPA6 bound to the extracellular matrix is enzymatically active [18]. The binding of CPA6 to the extracellular matrix can be blocked by pre-incubation with heparin, suggesting that CPA6 binds to regions of high negative charge density, possibly through cationic regions of the protein which are outward-facing. Binding to the extracellular matrix is one important factor that differentiates CPA6 from other CPA-like enzymes as well as most other peptidases in the M14 metallocarboxypeptidase family. An exception is CPZ, which binds to the extracellular matrix but preferentially cleaves basic residues and is therefore not likely to have any functional overlap with CPA6 [70,71].

The first connection between CPA6 and disease was made when a balanced translocation disrupting the gene was discovered in a patient with Duane Syndrome [72]. In this syndrome, the eye is not able to abduct (change direction and point away from the midline). Duane Syndrome is caused by defects in the lateral abducens muscle formation, development, or innervation. The CPA6 ortholog was knocked down in zebrafish using injections of morpholino oligonucleotides to block mRNA splicing. However, despite near total knockdown of CPA6 mRNA, no defects in the lateral abducens muscle, innervation of this muscle by cranial nerve VI or eye movement were observed [73]. No other phenotypes were apparent, and embryos were highly viable, suggesting that if knockdown of CPA6 had a phenotype in zebrafish embryos, it was not apparent under basal conditions.

Like other members of the CPA subfamily, CPA6 was predicted to cleave hydrophobic residues. However, carboxypeptidases vary widely in their selectivity for substrates, often being influenced by both the P1 and P1′ residues. For this reason, testing with conventional

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chromogenic substrates conjugated to a dipeptide often overlooks combinations that may be relevant to the enzyme's physiological function. Additional constraints such as peptide size, conformation or other properties will also not be examined using standard chromogenic assays. Peptidomics therefore played an integral role in testing the substrate specificity of CPA6 as several hundred endogenous brain peptides were tested for cleavage, using a scheme similar to that shown in Figure 2B. The results indicated that CPA6 preferentially cleaves bulky hydrophobic residues in the P1′ position, but cleavage efficiency is influenced by the residue in the P1 position [20]. Kinetic analysis with chromogenic substrates confirmed the importance of the P1 residue, validating the findings of the peptidomics experiment [20]. With the knowledge of what CPA6 is capable of cleaving, it is possible to predict whether an endogenous peptide could be a physiological substrate of CPA6.

The importance of finding CPA6 substrates was underscored when CPA6 mutations were found to be associated with epilepsy. In a study of a consanguineous family, it was shown that family members harboring homozygous Ala^{270} to Val mutations in CPA6 were affected with epilepsy and febrile seizures, while heterozygous siblings were unaffected [26]. This mutation was found to reduce the level of active enzyme present in the extracellular matrix to about 40% of the wild-type enzyme. This mutation did not affect activity per unit enzyme, leading to an overall decrease to 40% activity in these mutants. Subsequently, a second mutation that eliminated >95% of CPA6 from the extracellular matrix was identified in a temporal lobe epilepsy population, but not in a control population [26].

Bioinformatic analysis of single nucleotide polymorphisms reported in databases revealed some mutations that occurred at highly conserved residues, some of which caused substitutions of amino acids that altered the biochemical properties of that residue [27]. In addition, some single nucleotide polymorphisms found within the *CPA6* gene led to premature stop codons and truncation of the protein, eliminating critical active site residues required for enzymatic activity. Some mutations resulted in substitutions predicted to alter the structure or function of CPA6. These mutations were selected and analyzed using Pymol to examine their position. A group of mutations was selected for examination based on their presence in patients, likelihood to reduce CPA6 function or both. Assays of these mutations revealed several mutations present in human populations, which greatly reduced or eliminated CPA6 activity or expression in the extracellular matrix [27]. Several of these mutations have been identified in temporal lobe epilepsy but not control populations [27]. CPA6 is unlike other epilepsy genes that have been discovered to date, and the study of this peptidase may lead to important discoveries about the pathogenesis of epilepsy. This makes the identification of its endogenous substrates all the more interesting, as it is possible that the alteration of their cleavage in the absence of CPA6 underlies some rare forms of epilepsy.

Further studies must be carried out to test if loss of CPA6 in animals is sufficient to cause an increased susceptibility to seizures. This would verify that CPA6 mutations in epilepsy patients are loss of function. CPA6 knock-out animals would also be useful for peptidomic studies to identify substrates. In theory, substrates of CPA6 should increase, and products should decrease with the absence of CPA6. However, since ablation of CPA6 is expected to cause seizures, which themselves alter the levels of peptides and proteins in the brain, it is

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possible that other molecules will be altered that are not substrates of CPA6. Given that the substrate specificity of CPA6 is now established, it will be possible to compare what is altered in CPA6 knockout mice to what CPA6 is capable of cleaving, which should help determine the endogenous substrates.

CCP1/Nna1

Since its discovery in the 1970's [25] the purkinje cell degeneration (*pcd*) mouse has been the subject of many scientific investigations because of its characteristic ataxia. Although it has a complex phenotype, the neurons that degenerate in this mutant do so after ~1–3 months, making this a valuable model for uncovering genes and pathways related to adult-onset neuronal degeneration. In 2002, the *pcd* mutation was found to interrupt the gene encoding *Nna1* [24], which was so named because it was thought to be neuronal-specific, nuclear, and induced by axotomy [8]. Nna1 expression declined as nerves regenerated, but maintained a high level of expression in transected nerves that did not regenerate, suggesting a role in regulating regeneration of damaged neurons. Further, it was localized in differentiating but not proliferating neurons [8].

Nna1 was initially recognized as being a member of the M14 metallocarboxypeptidase family based on sequence homology, although the original alignments of Nna1 to other members of the family suggested that some of the critical active site residues were missing in Nna1[8]. A more thorough alignment of Nna1 showed that all of the critical active site residues in other members of the M14 metallocarboxypeptidase family were conserved in Nna1 [74] and in five novel members of the Nna1 subfamily [9]. Due to the expression of Nna1 in many non-neuronal cells and its predominant localization to the cytosol under basal conditions, the name cytosolic carboxypeptidase 1 (CCP1) was proposed to replace Nna1, with the five new members named CCP2 through CCP6 [9].

Because CCP1 is a large protein, containing N and C-terminal regions extending beyond the catalytic domain (Fig. 1), it wasn't clear if the neurodegeneration seen in the *pcd* mice was due to loss of enzymatic activity or another function of CCP1. This was tested by expressing various forms of CCP1 in the Purkinje cells of *pcd* mice. Whereas the *pcd* mutant mouse could be rescued by wild-type CCP1 mRNA, the phenotype was not rescued by mutant forms of CCP1 in which active site residues were altered [74]. This suggested that CCP1 was an active enzyme, and that the loss of its carboxypeptidase activity was responsible for the phenotype of the *pcd* mouse. Subsequently, CCP1 was purified and shown to be an active carboxypeptidase [12].

Initially, there were two possible functions proposed for CCP1 and related CCPs based on their localization to the cytosol and proteolytic events that occur in this compartment. One function involved the modification of tubulin, which is known to undergo C-terminal trimming of 1–2 residues (and very recently, of a third C-terminal residue) [12]. Furthermore, tubulin gets modified by the attachment of glutamate or glycine residues to the gamma carboxyl group on the side chain of a glutamate near the C-terminus, and then the gamma-linked residue is extended in alpha-linked chains. These steps are undone by one or more carboxypeptidases, and the CCPs were logical candidates. The other proposed function

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for the CCPs was a role in protein turnover [75]. Proteins are cleaved into peptides by the proteasome, a multi-subunit enzyme located in the cytosol. The resulting peptides then need to be rapidly degraded into amino acids by cytosolic peptidases, a process that is vital for protein turnover and to prevent the accumulation of peptides inside of the cell. Although it had been proposed that only aminopeptidases and not carboxypeptidases contribute to peptide turnover [76–79], there is no a priori reason for this. Therefore, it was important to test all potential functions for cytosolic carboxypeptidases.

A quantitative peptidomic technique was used to compare the levels of peptides in several *pcd* mouse brain regions with corresponding levels in wild-type mice, and it was found that *pcd* animals had dramatic increases in the levels of hundreds of peptides (such as the example shown in Fig. 2C, F)[75]. Moreover, levels of the major cellular proteins were not altered, so it was reasonable to conclude that the alteration was occurring in the steps required to convert peptides to amino acids. Additionally, *pcd* mice had elevated levels of autophagy [75,80]. If CCP1 was a major component of peptide breakdown, it would be logical to conclude that those cells would accumulate peptides and subsequently be deprived of amino acids, thus stimulating autophagy. However, these results came into question as subsequent peptidomic studies of young *pcd* mice (prior to the onset of neurodegeneration) showed no differences in peptide levels compared to wild-type mice [81]. Furthermore, peptidomic analysis of other adult tissues (heart, spleen) of *pcd* mice did not show any major changes in peptide levels of the mutant mice, compared to wild-type mice, unlike the results with adult brain [81]. The changes in peptide levels seen in adult *pcd* mouse brain occurred after the onset of the ataxic phenotype, and were most likely secondary effects.

In 2010, studies examining the effect of overexpression or knock-down of CCP1 and related CCPs found changes in the levels of glutamylated tubulin, as well as myosin light chain kinase [11]. Subsequent studies with purified CCP1 showed that the enzyme could directly remove glutamate residues from the C-terminus and side chain of tubulin [12]. These studies established CCP1 and other CCPs as protein deglutamylating enzymes, opposing the function of ligases, which add glutamates to the side chains of tubulin and other cellular proteins.

Summary and caveats

Cleavage of the C-term residues of peptides and proteins is important for diverse biological processes including activation, degradation and modulation of physiological activity. Loss or alteration of this process has been shown to be involved in many diseases such as obesity, anxiety, depression, infertility, neurodegeneration, and epilepsy. As more loss of function experiments are carried out, it is likely that other CPs will be implicated in pathology.

Linking C-terminal processing to physiological processes is challenging. Peptidomics can play an integral role in measuring the precise molecular changes that occur in the absence of CP activity. As the processing enzyme is lost, substrates should accumulate and products should not be formed (Fig. 2C, F). This was the case for many of the peptides observed in studies on *Cpe*$^{fat/fat}$ mice, with large increases in the levels of CPE substrates (i.e. peptide processing intermediates with C-terminal Lys/Arg residues) and large decreases in levels of

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CPE products (i.e. mature peptides). These results fit with the known specificity of CPE for basic residues and its localization to the secretory pathway. Peptidomics in these animals was a powerful way to discover many CPE substrates. However, not all of the results initially made sense; some of the peptides that were elevated in the *Cpe*$^{fat/fat}$ mice were not likely substrates of CPE based on the known specificity of this enzyme. The absence of an enzyme can lead to physiological changes that indirectly alter peptide levels. In *Cpe*$^{fat/fat}$ mice, endoproteases that recognize dibasic residues are inhibited by C-terminal Lys/Arg extended peptides that accumulate in the absence of CPE [82]. This indirectly leads to an increase of the substrates and decrease of the products of these endoproteases.

Another example of indirect effects comes from studies of the CCP1-deficient *pcd* mouse. Peptidomics studies of *pcd* mice showed large accumulations of cytosolic peptides, raising the possibility that CCP1 played a role in the degradation of these peptides. However, once CCP1 was purified and enzyme activity assayed, it was only found to cleave glutamate residues from proteins and peptides, and not to function as a general peptide-degrading enzyme. Because the vast majority of peptides elevated in the *pcd* mouse brain did not contain C-terminal glutamates, the increased levels of these peptides was not the direct result of deficient CCP1 activity. It remains to be shown why peptides are elevated in adult *pcd* mouse brain, but it is likely a secondary change resulting from neurodegeneration. This example of the *pcd* mouse underscores the importance of knowing the substrate specificity of an enzyme when interpreting peptidomics results. Additionally, because it is known that CCP1 and CCP5 process tubulin, and may process the C-termini of other polyglutamylated proteins, approaches that can detect carboxypeptidase cleavages of proteins (rather than peptides) will be important to study the biological roles of these enzymes.

Using peptidomics to determine substrate specificity (Fig. 2B) differs from peptidomics studies that aim to look for endogenous substrates (Fig. 2C). To examine substrate specificity, crude extract is incubated with purified enzyme and then analyzed by quantitative mass spectrometry. This method exposes the enzyme to many substrates, including some that the enzyme would never come into contact with in physiological conditions. This method does not necessarily provide information about all physiological substrates of an enzyme, and is instead a method to determine the substrate specificity with a large library of substrates, some of which may represent the true endogenous substrates. Similar methods using protein fragments have also been used to examine substrate specificity of proteolytic enzymes [21,83]; this can be helpful to increase the likelihood of finding substrates for highly specific enzymes [84].

In summary, peptidomics techniques are powerful approaches for studying CPs and other enzymes that are active against peptides. Studies testing purified enzymes with mixtures of peptides reveal what an enzyme can cleave, and studies of knockout animals reveal what changes in its absence. Taken together, an extensive library of substrates and products can be compiled, as has been done for CPE. The roles of these individual peptides can be studied subsequently to examine how the loss of the enzyme of interest causes a phenotype. This can have important implications if new signaling molecules can be discovered which are involved in processes relating to a pathology of interest. In the case of CPE, novel peptides were identified that have a role in feeding and body weight regulation [85].

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Acknowledgments

The authors gratefully acknowledge grant support from the National Institutes of Health grant DA-004494 (L.D.F.).

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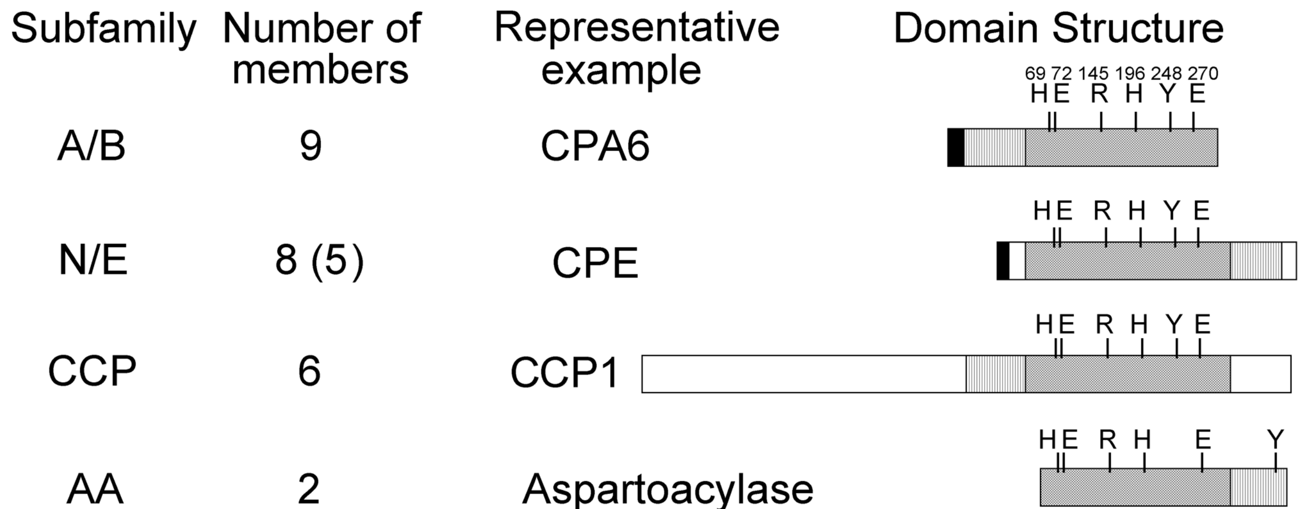

Figure 1. Subfamiles and Domain Structure of M14 Metallocarboxypeptidases

The M14 family of metallocarboxypeptidases is divided into four subfamilies differentiated by domain structure and homology. The enzymatic CP domain is shown with grey shading; this domain has amino acid sequence homology between various members of the family, whereas all other domains are only conserved within the subfamily and not between the different subfamilies. All active members of the M14 family contain the six conserved residues shown, which coordinate the central zinc ion (H69, E72, H196) and participate in substrate binding (R145, Y248) or catalysis (E270). As per convention in the field, the numbering system of these residues is based on their position in the mature form of bovine CPA1. All members of the A/B and N/E subfamilies contain an N-terminal signal peptide of ~20–30 amino acids (indicated by black boxes); none of the CCP or AA subfamily members contain this domain. All but one member of the CPA/B subfamily contain an inactivating prodomain of ~90 residues (indicated by shading with vertical lines), located N-terminal to the carboxypeptidase domain. This region is folded into a beta sheet-rich structure. Members of the N/E subfamily do not have this prodomain, but have C-terminal transthyretin-like domain thought to be involved in protein folding; although no sequence or structural homology to the prodomain of most A/B subfamily members, the transthyretin-like domain of the N/E subfamily members folds into a beta sheet-rich structure (indicated by vertical shading). There are a total of 8 members of the N/E subfamily, although only 5 of these have been shown to have carboxypeptidase activity; three other family members are not active as carboxypeptidases and lack one or more of the critical residues indicated in the figure. Members of the CCP family have several additional domains of unknown function; the length of these domains vary by family member. CCP1 has a large N-terminal domain and a shorter C-terminal domain. All CCPs contain an N-terminal domain that folds into a beta sheet-rich structure (indicated by vertical lines). Aminoacylases have a C-terminal shielding domain that limits accessibility to the active site. The C-terminal domain folds into a beta sheet-rich structure (indicated by vertical lines). In the aminoacylases, the residue that is functionally equivalent to Tyr 248 is located within this C-terminal domain, but in the three-dimensional structure folds into a conformation with an active site very similar to those found in other subfamilies.

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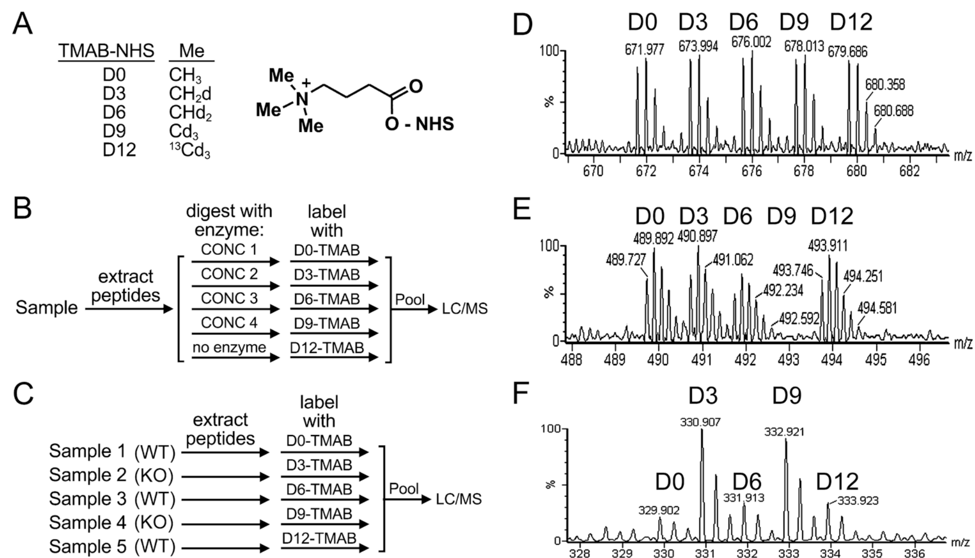

Figure 2. Quantitative peptidomic approaches to study carboxypeptidases

A. Chemical reagents used to label free amines on peptides. TMAB-NHS, trimethylammoniumbutyryl-N-hydroxysuccinimide; Me, methyl; d, deuterium. The five different isotopic forms of this compound are named D0, D3, D6, D9, and D12, although the latter compound contains 9 deuterium atoms, and one 13C for a 12 Dalton mass difference from the D0 form. **B**. Representative experiment to examine which native peptides can be cleaved by a peptidase. In this example, peptides are extracted from the biological sample and then divided into five aliquots, each of which is incubated with a different concentration of enzyme. Following the reaction, the peptides are labeled with the TMAB-NHS isotopic tags, pooled, and analyzed by liquid chromatography/mass spectrometry (LC/MS). **C**. A representative experiment to examine which peptides are altered by the absence of an enzyme in knockout (KO) animals. A similar approach can be used with knockdown or overexpression techniques. In the example shown, three wild-type animals (WT) are compared to two KO animals. Peptides are extracted from the tissues, labeled with the TMAB-NHS tags, pooled and analyzed by LC/MS. **D**. Representative data showing a peptide that is neither a substrate nor product of the enzyme. This spectrum was from an experiment testing different concentrations of carboxypeptidase D with the cellular peptidome from HEK293T cells. The peptide was subsequently identified by tandem mass spectrometry as an internal fragment of 60S acidic ribosomal protein P2 (sequence LDSVGIEADDDRLNKV) with two TMAB tags incorporated and 1 proton (total charge 3+). Although this spectrum was from the scheme shown in panel B, a similar pattern would be observed for studies using the scheme shown in panel C for peptides that are neither substrates nor products. **E**. Representative data showing a peptide that is a substrate for carboxypeptidase D, from the same experiment as in panel D, using the scheme shown in Panel B. The peptide was subsequently identified by tandem mass spectrometry as a C-terminal fragment of 40S ribosomal protein S28 (sequence KGPVREGDVLTLLESEREARRLR) with 2 TMAB tags and 4 protons (total charge 6+). Note the dramatic decrease in peak height with the highest concentration of enzyme (D9) and a moderate decrease in peak height with the second highest concentration of enzyme

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(D6). **F**. Representative data showing a peptide that is elevated in the striatum of adult mice lacking active CCP1 due to a naturally occurring mutation (*pcd*^{3J}). This peptide was not identified by tandem mass spectrometry. In the original report describing the changes in the peptidome in the adult *pcd* mouse brain, peptides that were elevated were thought to be substrates of CCP1 [75]. However, studies testing purified CCP1 with substrates showed a specificity for C-terminal Glu residues and no activity against peptides with C-terminal hydrophobic residues, whereas most of the peptides elevated in the peptidomics analysis of *pcd* mice contained C-terminal aliphatic residues [11–13]. Further peptidomic analysis of *pcd* mouse tissues showed that levels of peptides were not altered in the brains of young animals, taken prior to the onset of neurodegeneration [81]. Thus, while the result in panel F is consistent with the change expected for a CCP1 substrate, it is more likely an indirect change that results from the neurodegeneration of cerebellar Purkinje cells. This emphasizes the need to perform both types of studies; those testing the activity of purified enzymes with mixtures of peptides (as in Panels B, D, and E) and those testing animals lacking the enzyme (as in Panels C and F).