

Characterization of the Substrate Specificity of Human Carboxypeptidase A4 and Implications for a Role in Extracellular Peptide Processing^{*[5]}

Received for publication, August 27, 2009, and in revised form, April 9, 2010. Published, JBC Papers in Press, April 12, 2010, DOI 10.1074/jbc.M109.060350

Sebastian Tanco[‡], Xin Zhang[§], Cain Morano[§], Francesc Xavier Avilés[‡], Julia Lorenzo^{‡1}, and Lloyd D. Fricker^{§2}

From the [‡]Institut de Biotecnologia i de Biomedicina and Departament de Bioquímica, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain 08193 and the [§]Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

CPA4 (carboxypeptidase A4) is a member of the metallo-carboxypeptidase family. CPA4 was originally found in a screen of mRNAs up-regulated by sodium butyrate-induced differentiation of cancer cells. Further studies suggested a relation between CPA4 and prostate cancer aggressiveness. In the present study, we determined that CPA4 is secreted from cells as a soluble proenzyme (pro-CPA4) that can be activated by endoproteases, such as trypsin. Three complementary approaches were used to study the substrate specificity of CPA4; kinetic analysis was performed using a new series of chromogenic substrates and some biologically relevant peptides, the cleavage of synthetic peptides was tested individually, and the cleavage of a mixture of >100 mouse brain peptides was examined using a quantitative peptidomics mass spectrometry-based approach. CPA4 was able to cleave hydrophobic C-terminal residues with a preference for Phe, Leu, Ile, Met, Tyr, and Val. However, not all peptides with C-terminal hydrophobic residues were cleaved, indicating the importance of additional residues within the peptide. Aliphatic, aromatic, and basic residues in the P1 position have a positive influence on the cleavage specificity. In contrast, acidic residues, Pro, and Gly have a negative influence in the P1 position. Some of the peptides identified as CPA4 substrates (such as neurotensin, granins, and opioid peptides) have been previously shown to function in cell proliferation and differentiation, potentially explaining the link between CPA4 and cancer aggressiveness. Taken together, these studies suggest that CPA4 functions in neuropeptide processing and regulation in the extracellular environment.

Carboxypeptidases (CPs)³ hydrolyze a single amino acid from the C terminus of peptides and proteins. Metallo-CPs use

^{*} This work was supported in part by National Institutes of Health Grants DK-51271 and DA-04494 (to L. D. F.). This work was also supported by European Commission Grant FP7-Health-2009-241919-LIVIMODE (to F. X. A.), by Spanish Ministry of Science and Technology Grant BIO2007-68046-C02 (to F. X. A.), and by Xarxa de Referència en Biotecnologia (Generalitat de Catalunya).

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S4 and Fig. S1.

¹ To whom correspondence may be addressed. Tel.: 34-935811231; Fax: 34-935812011; E-mail: julia.lorenzo@uab.es.

² To whom correspondence may be addressed: Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-4225; Fax: 718-430-8922; E-mail: lloyd.fricker@einstein.yu.edu.

³ The abbreviations used are: CP, carboxypeptidase; CPA, A-like carboxypep-

a catalytic mechanism in which nucleophilic attack on a peptide bond is mediated by a Zn²⁺-activated water molecule (1). Most metallo-CPs are classified based on amino acid sequence, structure, and function into subfamilies within clan MC, family M14 (2). These subfamilies include M14A, also known as the CPA/CPB subfamily; M14B, also known as the CPN/CPE subfamily; M14C, which includes γ -D-glutamyl-(L)-meso-diaminopimelate peptidase I from *Bacillus sphaericus*; and a proposed fourth subfamily, including cytosolic CPs (3, 4). Metallo-CPs have also been divided into subgroups based on substrate specificity: A-like enzymes with preference for hydrophobic C-terminal amino acids; B-like enzymes, which cleave C-terminal basic residues; CPs with preference for glutamate in the C-terminal position; and CPs with a broad substrate specificity.

CPA4 (carboxypeptidase A4) belongs to the M14A subfamily of carboxypeptidases. The pancreatic members of this subfamily (CPA1, CPA2, and CPB) act in the degradation of dietary proteins in the digestive tract. Other members of this subfamily display a wide spectrum of physiological roles. Mast cell carboxypeptidase (recently renamed CPA3) is found in the secretory granules of mast cells and plays a role in defense (5). CPA6 is linked to Duane syndrome, a congenital eye movement disorder, and has been proposed to participate in the regulation of neuropeptides in the extracellular matrix within the olfactory bulb and other parts of the brain (6). Carboxypeptidase U, also known as CPB2 and TAFI (thrombin-activatable fibrinolysis inhibitor), participates in fibrinolysis inhibition and possesses anti-inflammatory properties (7) and for these reasons is of interest to the pharmaceutical industry (8).

CPA4 was originally referred to as CPA3 (9) but was renamed CPA4 to reflect the order in which the CPAs were discovered. Huang *et al.* (9) identified CPA4 in a search for mRNAs induced by sodium butyrate in androgen-independent prostate cancer cells. The histone deacetylase inhibitor trichostatin A also induced the expression of CPA4 mRNA in PC-3, DU145, and BPH1 human prostate cancer cell lines (9). Huang *et al.* (9) also reported that CPA4 mRNA expression is associated with hormone-regulated tissues, suggesting that it may have a role in cell growth and differentiation. The human *CPA4* gene is located

tidase; CPB, carboxypeptidase B; ECM, extracellular matrix; ER, endoplasmic reticulum; LC, liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS/MS, tandem mass spectrometry; TMAB, 4-trimethylammoniumbutyrate; HA, hemagglutinin; FA, 3-(2-furyl)acryloyl.

Characterization of Human Carboxypeptidase A4

on chromosome 7q32, which is a region in the genome that might contain genes for prostate cancer aggressiveness (10). In addition, the *CPA4* gene was found to be maternally imprinted in a tissue-specific manner (11, 12). Furthermore, imprinting in adult benign hypertrophic prostate tissue suggests that mutations or aberrant imprinting in *CPA4* may be related to prostate cancer aggressiveness (12). Ross *et al.* (13) studied the association of single-nucleotide polymorphisms on the *CPA4* gene and its relation to prostate cancer and found that the nonsynonymous coding single-nucleotide polymorphism (G303C) rs2171492 was associated with an increased risk of aggressive disease in younger men.

Despite the potential importance of CPA4 toward prostate and other cancers, no previous studies have examined the substrate specificity of CPA4 in detail. The three-dimensional structure of CPA4 has been determined for the zymogen state (14) and for the active form in complex with latexin, a brain-derived CP inhibitor (15), and in complex with a hexapeptide (16). This latter study also compared the cleavage of a handful of synthetic peptides by CPA4, but no kinetic details were provided. To gain a better understanding of the enzymatic properties of CPA4, we used a variety of approaches to characterize the substrate specificity. A series of dipeptide chromogenic substrates were synthesized and tested with purified CPA4. A number of synthetic peptides were individually tested with purified CPA4; many of these peptides correspond to biologically active peptides. A third approach involved incubating CPA4 with a mixture of over 100 endogenous peptides extracted from mouse brain, using a quantitative peptidomics approach to identify substrates and products. The subcellular distribution, secretion, and pH optimum of CPA4 were also examined. Taken together, these studies provide a complementary and thorough analysis of the cleavage specificity of CPA4 and suggest that this enzyme functions as a soluble extracellular peptidase that removes C-terminal hydrophobic/aliphatic residues from secreted peptides.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—Human pro-CPA4 (PCPA4) was produced using the vector pPIC9 and the methylotrophic yeast *Pichia pastoris* as an expression host and purified as described elsewhere (15). The active enzyme was obtained through tryptic activation (at a 1:10 w/w ratio) for 60 min at room temperature, and the resulting product was subsequently purified by anion-exchange chromatography (TSK-DEAE 5PW) using an FPLC-Äkta system with a linear salt gradient from 0 to 30% of 0.4 M ammonium acetate in 20 mM Tris-HCl (pH 10.5). Eluted fractions were analyzed by SDS-PAGE, and the purest samples containing the active enzyme were pooled, desalted, and concentrated to 1 mg/ml by Amicon centrifugal filter devices.

Cell Culture and Transfection—HeLa (human cervix adenocarcinoma), PC-3, DU145, and LNCaP (all three cell lines are human prostate carcinoma), 1BR3.G (human skin fibroblasts), and HEK 293T (human embryo kidney) cells were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in the recommended growth medium: Eagle's minimum essential medium or Dulbecco's modified

Eagle's medium with 4500 mg/liter glucose, GlutaMAX-I, and pyruvate supplemented with 10% fetal calf serum (Invitrogen). The full-length cDNA sequence of human pro-CPA4 was cloned into pcDNA3.1 vector (Invitrogen), and the hemagglutinin (HA) epitope was introduced on the C terminus of CPA4. The plasmid pDsRed2-ER from Clontech was used for endoplasmic reticulum (ER) localization. Cells were grown in 35-mm dishes and transfected with Lipofectamine 2000 according to the manufacturer's instructions.

Cell Fractionation and Processing—Forty-eight hours after transfection, medium was removed, centrifuged, and collected on ice. The dishes were gently washed with phosphate-buffered saline, and cells were removed by pipetting up and down in a mild extraction buffer consisting of 50 mM Tris-HCl, pH 8.0. The cell suspension was sonicated and centrifuged at $16,000 \times g$ for 10 min at 4 °C. The pellet was resuspended in an equivalent amount of 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl and 1% Nonidet P-40 and centrifuged as above to produce a detergent/high salt extract. Following the removal of cells, the plate was washed several times with phosphate-buffered saline, and the extracellular matrix (ECM) was extracted by adding hot SDS-PAGE sample buffer directly to the plate. All homogenates were frozen and stored at -70 °C until analysis. For trypsin activation assays, 200 ng of trypsin (Sigma) were added to 20 μ l of the extracellular medium of transfected HEK 293T cells and incubated for 30 min at 37 °C.

Immunoblot—For immunoblotting, equivalent amounts of each lysate or fraction were analyzed by SDS-10% PAGE, and the separated proteins were electrophoretically transferred onto polyvinylidene difluoride filters (Millipore Corp.). Non-specific binding sites on the polyvinylidene difluoride filters were blocked by incubation with 5% skim milk for 12 h. The polyvinylidene difluoride filters were then incubated with anti-HA antibody (dilution 1:3,000; Sigma) for 1 h at 25 °C. After washing with phosphate-buffered saline containing 0.05% Tween 20, the filters were incubated with goat anti-mouse IgG-peroxidase (dilution 1:10,000; Pierce) for 1 h. After three rinses, immunoreactive bands were visualized with a chemiluminescence detection kit (ECL; Millipore).

Immunofluorescence—HeLa, HEK 293T, PC-3, DU145, or LNCaP cells were co-transfected with HA-tagged PCPA4 and pDsRed2-ER using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h of incubation, cells were fixed in methanol, permeabilized with 0.1% Triton X-100, and incubated with anti-HA antibody (clone HA-7 (Sigma); 1:2,000 dilution), followed by conjugated Alexa-488 anti-mouse-IgG (Molecular Probes; 1:500 dilution) and 4',6'-diamidino-2-phenylidole. Coverslips were mounted with Fluoprep mounting medium (Biomerieux) and analyzed with a confocal laser-scanning microscope (Leica Microsystems, Bannockburn, IL).

Substrate Synthesis—Chromogenic substrates were synthesized according to Blumberg *et al.* (17). 3-(2-furyl)acryloyl (FA)-Phe-*N*-hydroxysuccinimide was prepared according to the following procedure. A solution of 15 mmol of FA-Phe and 15 mmol of *N*-hydroxysuccinimide in 60 ml of dioxane-dimethylformamide (1:1) was cooled to 4 °C, and 15 mmol of dicyclohexylcarbodiimide was added. The solution was stirred at 4 °C

for 16 h. The dicyclohexyl urea was filtered, and the solution was evaporated *in vacuo*. Furylacryloyl dipeptides were prepared by reacting FA-Phe-*N*-hydroxysuccinimide with the appropriate amino acid according to the following procedure. The *N*-hydroxysuccinimide ester (5 mmol) in 25 ml of dioxane was added at room temperature to a solution of the amino acid (6 mmol) and sodium bicarbonate (12 mmol) in 25 ml of water. The mixture was kept at room temperature for 6 h, the dioxane evaporated, and the residue acidified with 1 N HCl. After the mixture was cooled overnight at 4 °C, the precipitate was collected by filtration and dried.

Kinetic Measurements—The rate of hydrolysis of different substrates was continuously measured at 25 °C in 50 mM Tris, 0.15 M NaCl at the optimal pH for each enzyme. The wavelengths varied slightly among substrates: 336 nm for FA-Phe-Phe, FA-Phe-Ile, and FA-Phe-Trp; 338 nm for FA-Phe-Ala, FA-Phe-His, and FA-Phe-Leu; and 340 nm for FA-Phe-Val and FA-Phe-Met. Initial rates determined from the first 5–10% of the time trace of each reaction were obtained at substrate concentrations close to the K_m value whenever possible. The kinetic parameters, k_{cat} and K_m , were obtained using six experimental points by direct fit to a Michaelis-Menten curve using a nonlinear least-squares regression analysis. For those substrates for which the solubility of the substrates limited the measurements to concentrations well below K_m , the k_{cat}/K_m values were estimated from the slope of the linear portion of saturation curve using the simplified equation, $v = (k_{cat}/K_m) \cdot [E_0] \cdot [S]$.

Measurement of Equilibrium Dissociation Constants (K_i)—The method for reversible tightly binding inhibitors described by Bieth (18) was used for K_i determination. Recombinant purified CPA4 at a concentration of 8 nM was assayed against increasing concentrations of inhibitor. Initial rates, determined from the first 5–10% of the time trace of each reaction, were obtained for each inhibitor concentration using the *N*-(4-methoxyphenylazofonyl)-L-phenylalanine substrate (Bachem). K_i values were obtained by direct fitting of fraction velocity versus inhibitor concentration data to the Morrison equation using the Grafit program.

Substrate Specificity and Kinetic Measurements Using Peptides—CPA4 substrate specificity was analyzed using synthetic peptide substrates. Reactions were performed in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, at 37 °C using peptide and enzyme amounts of 10 μ mol and 1 nmol, respectively. Peptide cleavage reactions were stopped at different times (30 min, 1 h, 2 h, and 16 h) by dropping the pH to 4 using 1% trifluoroacetic acid. The reaction products were desalted using a C18 ZipTip (Millipore), following the manufacturer's protocol. The peptides were eluted with 1 μ l of 70% acetonitrile, 0.1% trifluoroacetic acid; mixed with an equal volume of a saturated solution of α -hydroxycinnamic acid in the same solvent; and analyzed in a Bruker Daltonics Ultraflex matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer.

For the determination of kinetic constants for the hydrolysis of peptides, rates of substrate hydrolysis were determined by reversed-phase high performance liquid chromatography in a Waters Alliance apparatus using a Grace Vydac 218TPTM C18 column (3 μ m, 100 Å, 4.6-mm inner diameter \times 250 mm; Hes-

peria, CA) with a linear gradient of 5–35% acetonitrile and following the absorbance at either 214 or 280 nm. Reactions were performed at 37 °C for 30 min in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, and stopped by the addition of 0.1% trifluoroacetic acid. Substrate concentrations ranged from 0.2 to 5 times the K_m whenever possible, and the enzyme concentration was chosen so that hydrolysis was less than 10% of product formed. The kinetic parameters, k_{cat} and K_m , were obtained by direct fit to a Michaelis-Menten curve using the Grafit program.

Peptidomics—Peptides were extracted from whole mouse brain (minus the olfactory bulb and cerebellum) as described (19, 20). In brief, four mice (C57B6/J, 2–3 months old) were sacrificed by decapitation, and the head was immediately irradiated in a conventional microwave oven for 8 s to raise the brain temperature to 80 °C and inactivate proteases that can rapidly degrade cellular proteins during postmortem processing of the tissue (21). The mouse brain was isolated, the olfactory bulb and cerebellum were discarded, and the remainder of the brain was frozen in dry ice and stored at -70 °C until peptide extraction. Peptides were extracted by sonication of each brain in 1.0 ml of ice-cold water followed by incubation at 70 °C for 20 min, cooling in an ice bath, acidification with 120 μ l of 0.1 M HCl to a final concentration of 10 mM HCl, and centrifugation at $13,000 \times g$ for 30 min at 4 °C. The supernatants (peptide extracts) from each of the four mice were pooled and mixed, and 60- μ l aliquots were neutralized to pH 8 by 75 mM borate buffer and incubated in the presence of purified CPA4 (20 ng, 670 pg, or 22 pg) or in the absence of enzyme for 90 min. The reactions were stopped by the addition of 2.5 mM benzylsuccinic acid and heat-inactivated at 80 °C for 10 min. Labeling with 4-trimethylammoniumbutyrate (TMAB) isotopic tags containing either no deuterium (D0) or 3 atoms (D3), 6 atoms (D6), or 9 atoms (D9) of deuterium was performed as described (19). After labeling, the TMAB reagent was quenched with glycine, the four samples were combined, and peptides were isolated by passage through a Microcon[®] YM-10 unit (Millipore) to remove proteins of >10 kDa. The samples were treated with hydroxylamine to remove TMAB labels from Tyr residues, desalted with a PepCleanTM C-18 spin column (Pierce), and concentrated to ~ 20 μ l in a vacuum centrifuge.

Mass spectrometry was performed as described previously (19, 22). In brief, the peptide mixture was trapped and washed on a PepMapTM C18 trapping column (5 μ m, 100 Å, 300- μ m inner diameter \times 5 mm, LC Packing (Marlton, NJ)) and separated on a Grace Vydac MS C18 capillary column (3 μ m, 100 Å, 75- μ m inner diameter \times 150 mm; Hesperia, CA) at 4 μ l/min with an acetonitrile gradient in 0.1% formic acid. The column eluate was analyzed on an API Q-Star Pulsar-*i*TM quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) in information-dependent acquisition mode, with a collision energy for tandem mass spectrometry (MS/MS) (20–45 eV) based on the m/z value and charge state of the ion selected.

Data were analyzed as described (19). In brief, Mascot (available from the Matrix Science Web site) was used, followed by manual verification; identifications were rejected unless 80% or more of the major fragments observed in MS/MS matched predicted b- or y-series fragments (minimum of five matches).

Characterization of Human Carboxypeptidase A4

Additional criteria included all of the following: a parent mass within 40 ppm (preferably 20 ppm) of the theoretical mass; the observed number of TMAB tags on the peptide matching the predicted number of free amines available (*i.e.* Lys residue and N terminus); and the observed charge state(s) of the peptide consistent with the expected number of positive charges. The intensities of the TMAB-labeled isotopic peaks were determined by measuring peak heights of the monoisotopic peak and the additional peaks containing one and two atoms of ^{13}C . The peak intensities of the samples incubated with enzyme were compared with the peak intensity of the sample incubated without enzyme; previous studies examining four aliquots of brain extract with the four different isotopic tags showed average ratios of 1.00 (19).

RESULTS

The sequence analysis of PCPA4 protein reveals the presence of a 16-amino acid-long signal peptide consensus sequence, and therefore the protein is predicted to be translocated to the lumen of the ER upon synthesis. From the ER, many options are possible, including retention in the secretory pathway, targeting to lysosomes, or secretion. Because CPA4 lacks any transmembrane domains and other members of this subfamily are secreted, PCPA4 is predicted to also be secreted from cells. Some CPs, such as CPA1, remain as soluble proteins in the extracellular environment (23), whereas others like CPA6 (6) or mouse CPZ (24) are retained within the ECM. In relation to proprotein processing, some pro-CPs are activated before or upon secretion by proteases present in the secretory pathway (6), whereas others are activated after secretion by extracellular proteases (23). To study whether PCPA4 is secreted and activated, HEK 293T cells were transiently transfected with HA-tagged PCPA4 cDNA. After 48 h of transfection, cells, media, and ECM were collected, fractionated, and analyzed by Western blot. A band of an apparent molecular mass of 50 kDa corresponding to the full-length PCPA4 was detected predominantly in the cell culture medium (Fig. 1A, lane 5). PCPA4 was also detected in two different cell extracts; the zymogen appears in a low salt extract but also at low levels in a detergent/high salt extract (Fig. 1A, lanes 1 and 3). The absence of any PCPA4 in the ECM shows that the protein remains as a soluble protein in the extracellular medium. In all cases, PCPA4 is found in the full-length form, indicating that its activating protease is not present or active in sufficient levels in the system. To investigate if this behavior would be similar in other cell lines, we repeated the transfection of PCPA4 in five different cell lines: DU145 and PC-3, which are prostate cancer cells previously found to express PCPA4 in relatively high levels (9); LNCaP, a prostate cancer cell line that does not express PCPA4; HeLa cells, which arise from a cervical adenocarcinoma; and 1BR.3.G, a human skin fibroblast cell line. In all cases, PCPA4 was predominantly found soluble in the extracellular medium in the zymogen form (data not shown).

To demonstrate that PCPA4 expressed in HEK 293T cells was properly folded and able to produce the enzymatically active form, extracellular medium collected after 48 h of transfection was treated with trypsin. Western blot analysis of these samples shows that trypsin causes the disappearance of the 50

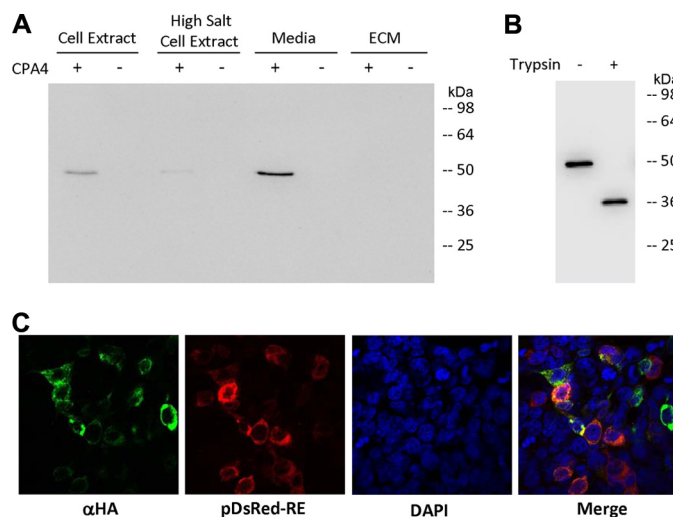


FIGURE 1. PCPA4 is a soluble secreted protein. A, PCPA4 transfection in mammalian cells. HEK 293T cells were transfected with empty vector (–) or plasmid expressing HA-tagged PCPA4 (+), and different fractions were collected and prepared. Equal amounts of each fraction were analyzed by Western blot using an HA antibody. B, trypsin processing. Extracellular media was treated with (+) or without (–) trypsin and analyzed by Western blot. C, subcellular distribution of PCPA4. HEK 293T cells were transfected with HA-tagged PCPA4 and pDsRed-ER cDNAs. After fixation, transfected cells were incubated with mouse anti-HA antibody, followed by anti-mouse IgG Alexa 488 (green) and 4',6'-diamidino-2-phenylindole (DAPI) to detect cell nuclei.

kDa band corresponding to PCPA4 and the appearance of a 35 kDa band corresponding to the expected molecular weight of the active enzyme (Fig. 1B). Enzymatic analysis of these samples shows no detectable CP activity in the extracellular medium of PCPA4-transfected cells, but incubation with trypsin resulted in the appearance of A-type CP enzymatic activity against the *N*-(4-methoxyphenylazoformyl)-L-phenylalanine substrate (data not shown). The finding that PCPA4 can be activated with trypsin indicates that it is properly folded and that the absence of detectable zymogen processing in the HEK 293T cells as well as in various other cell lines is due to insufficient levels of the activating enzyme and not due to the improper folding of PCPA4 in these cell lines.

The presence of PCPA4 in the cells was further investigated by immunocytochemistry. PCPA4 was found to show some overlap with an ER marker (Fig. 1C), which is consistent with the finding that intracellular PCPA4 is present in the secretory pathway of the cell.

Although there are several reports about CPA4 structure (14, 15), expression (9), and relation to prostate cancer aggressiveness (11, 12), a detailed analysis of its enzymatic properties has not been reported previously. Recombinant protein obtained in the *P. pastoris* system as described previously (15) was used for this purpose. Mature CPA4 was obtained after treatment of the proenzyme with trypsin and subsequent purification. As expected, the active enzyme shows the ability to remove C-terminal residues from both FA-Phe-Phe and FA-Phe-Ala. No detectable activity toward substrates with basic (FA-Ala-Arg) or acidic (hippuryl-L-glutamic acid) C-terminal residues was found. This is in accordance with predictions based on its sequence and structure that include CPA4 in the group of A-type CPs. The *Pichia*-expressed proenzyme shows 3% of

residual activity against the *N*-(4-methoxyphenylazobenzyl)-*L*-phenylalanine substrate when compared with the active enzyme. Although the proenzyme shows residual activity, it could not be detected in the conditioned medium of PCPA4-transfected HEK 293T cells because this activity falls below the limit of detection of the technique.

The influence of pH on CPA4 activity was analyzed using both FA-Phe-Phe and FA-Phe-Ala. The optimal pH for cleavage of either substrate is in the range of 8.5–9 with very low activity below pH 6 (Fig. 2). This suggests that CPA4 is maximally active in the extracellular environment after secretion and has little activity within the acidic environment of the secretory pathway.

The effect of several different CP inhibitors on CPA4 activity was assayed. Benzylsuccinic acid (25), a potent synthetic inhibitor of the A-type CP subfamily, at a concentration of 100 μM produced 30% inhibition of CPA4 activity and completely inhibited the enzyme when a 1 mM concentration of the inhibitor was used. The Zn^{2+} -chelating agent 1,10-phenanthroline (26) showed 70% inhibition of the CP activity after 30 min of incubation at 1 mM concentration. CPA4 showed resistance against the action of EDTA, another chelating inhibitor of CPs; complete inhibition of CPA4 activity was only achieved using a 100 mM concentration of the inhibitor in an overnight incubation, whereas the same inhibitor concentration caused only

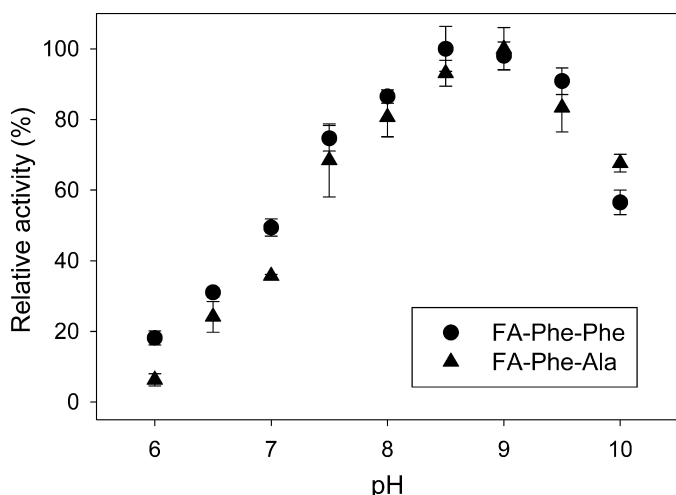


FIGURE 2. **Effect of pH on CPA4 activity.** CPA4 activity was determined by measuring initial rates using 100 μM FA-Phe-Phe or FA-Phe-Ala in 50 mM Tris acetate buffer at the indicated pH at 25 °C. Error bars, S.E. for three independent determinations.

TABLE 1
Kinetics constants for peptide substrate hydrolysis by CPA1, CPA2, and CPA4

Substrate	CPA4				CPA1				CPA2			
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}/K_m relative to FA-Phe-Phe	k_{cat}	K_m	k_{cat}/K_m	k_{cat}/K_m relative to FA-Phe-Phe	k_{cat}	K_m	k_{cat}/K_m	k_{cat}/K_m relative to FA-Phe-Phe
	s^{-1}	μM	$\text{M}^{-1}\text{s}^{-1}\cdot 10^{-5}$		s^{-1}	μM	$\text{M}^{-1}\text{s}^{-1}\cdot 10^{-5}$		s^{-1}	μM	$\text{M}^{-1}\text{s}^{-1}\cdot 10^{-5}$	
FA-Phe-Phe	44.3	55.6	7.97	1.00	76.6	20.4	37.6	1.00	121	36.4	33.3	1.00
FA-Phe-Trp	57.3	615	0.93	0.12	81.3	166	4.90	0.13	65.3	16.3	40.2	1.21
FA-Phe-Leu	13.4	19.4	6.93	0.87	34.0	15.7	21.7	0.58	18.6	393	0.47	0.014
FA-Phe-Ile	12.4	23.3	5.32	0.67	9.77	9.33	10.5	0.28	5.37	460	0.12	0.0035
FA-Phe-Met	23.9	40.0	5.81	0.73	51.8	128	4.03	0.11	NM ^a	NM	0.040	0.0012
FA-Phe-Ala	24.3	372	0.65	0.082	78.0	300	2.58	0.069	NM	NM	NM	NM
FA-Phe-Val	19.4	57.3	3.38	0.42	12.3	8.95	14.8	0.39	NM	NM	0.030	0.0010
FA-Phe-His	NM	NM	0.27	0.034	NM	NM	1.04	0.028	NM	NM	NM	NM

^a NM, not measurable; below the limit of detection.

60% inhibition after 5 h of treatment. Recombinant forms of potato CP inhibitor (27), leech CP inhibitor (28), and tick CP inhibitor (29) were tested with CPA4 and found to exhibit equilibrium dissociation constant (K_i) values in the low nanomolar range (supplemental Table S1). The protein inhibitor with the highest affinity for CPA4 was found to be that from tick *Rhipicephalus bursa*, for which a constant of 0.8 ± 0.3 nM was obtained (supplemental Table S1). The K_i values for CPA4 determined in the present study are generally comparable with values previously published for CPA4 with latexin (15) and *Ascaris* carboxypeptidase inhibitor (30); these results are included in supplemental Table S1 for comparison with the present data.

To investigate the substrate specificity of CPA4, a new series of substrates of the type FA-Phe-Xaa were synthesized. These substrates incorporated the furylacryloyl moiety as a chromophore, allowing for continuous monitoring of CP activity at a wavelength that has little interference from proteins or aromatic derivatives (31). This new series of substrates provided a broad range of hydrophobic/aliphatic residues in the C-terminal position so that substrate specificities of A-type CPs could be evaluated. The different amino acids included in the C-terminal position were His, Leu, Ile, Phe, Trp, Ala, Val, and Met. All substrates contain Phe in the penultimate position and are of the same length, allowing a direct comparison of the C-terminal residues of the substrates. Kinetic constants were determined for CPA4 and also for human CPA1 and CPA2 to directly compare these related enzymes with the same substrates. CPA1 and CPA2 were obtained as recombinant proteins in the *P. pastoris* system, purified in their zymogen form, activated with trypsin, and further purified as described previously (32, 33). k_{cat} , K_m , and k_{cat}/K_m values are shown in Table 1 and indicate that although all three enzymes have overlapping specificities, they differ markedly. Although CPA1 and CPA4 display similar and broader substrate specificities, CPA2 shows a substrate specificity that is essentially restricted to large aromatic residues in the C-terminal position. CPA4 shows a preference for hydrophobic C-terminal amino acids like Phe, Leu, Met, Val, and Ile, as judged by k_{cat}/K_m values. Of all of the dipeptide substrates tested with CPA4, FA-Phe-Trp exhibited the highest k_{cat} , but the high K_m resulted in a lower k_{cat}/K_m for this substrate than for FA-Phe-Phe. Overall, the k_{cat} varied only 5-fold among the substrates that showed measurable

Characterization of Human Carboxypeptidase A4

k_{cat} values, whereas the K_m varied 32-fold, and the k_{cat}/K_m varied 12-fold for these substrates (Table 1).

To further characterize the catalytic activity of CPA4, its ability to cleave a series of peptides was studied. Some of the peptides chosen for this analysis are biologically active and are known to be secreted into the extracellular fluid of tissues where CPA4 is expressed. Others are not likely to be biological substrates of CPA4 but provide further information about the ability of this enzyme to cleave peptides. All of these peptides were previously tested against CPA6 (6), so they are useful for comparison purposes. The enzyme, at a concentration of 1 nM, was incubated at 37 °C with the peptides in a final concentration of 10 μM . In order to obtain semiquantitative information about the cleavage, different times of incubation were analyzed by MALDI-TOF MS. Although some peptides were extensively cleaved, other peptides were only partially cleaved, and others were not cleaved under the conditions tested (supplemental Table S2). CPA4 was found to completely cleave PEN and Met-enkephalin-Arg-Phe after 30 min of incubation. Big SAAS and neurotensin were also extensively cleaved after 1–2 h of incubation. Partial cleavage of $\sim 50\%$ of angiotensin was found after 30 min of incubation. Des-Asp¹-angiotensin and Leu-enkephalin were partially cleaved in 2 h, and Met-enkephalin and CPA5 C terminus required an overnight incubation for a partial digestion by CPA4. Only a small percentage of cleavage was detected for human little SAAS. No digestion of the aldolase C-terminal peptide was detected, although it was expected to be cleaved because of the C-terminal Tyr. When a 10-fold higher concentration of enzyme (10 nM) was used, substantial cleavage of the C-terminal Tyr was observed after 2 h of incubation. Neither bradykinin nor des-Arg⁹-bradykinin were substrates for CPA4 (supplemental Table S2). Although des-Arg⁹-bradykinin has a C-terminal Phe, the presence of a Pro in the penultimate position may explain why CPA4 is not able to cleave this substrate under the conditions tested.

By using higher concentrations of enzyme (20 nM) and substrate (50 μM), it was possible to examine the processivity of CPA4 cleavage. The sequential hydrolysis of amino acids from the C terminus of neurotensin was observed (Fig. 3). The product of the cleavage of the C-terminal Leu was detected at 10 min of incubation whereas hydrolysis of the penultimate Ile was observed at 1 h of incubation (Fig. 3). Further removal of the Tyr was not detected, even upon prolonged incubation, presumably because of the negative influence of the Pro in the P1 position of this peptide (Fig. 3). The action of CPA4 (20 nM) with angiotensin (200 μM) also showed processivity; the removal of C-terminal Leu, penultimate His, and antepenultimate Phe is sequentially observed at 10 min, 30 min, and 4 h of incubation, respectively (data not shown).

Five of the peptides identified as CPA4 substrates were further investigated by determining the kinetic constants for CPA4 catalyzed hydrolysis. The data are summarized in Table 2 and show a broad variation in K_m and k_{cat} values for the different peptides. CPA4 cleaves neurotensin and Met-enkephalin-Arg-Phe with high efficiency (43.8 and 9.43 $\mu\text{M}^{-1} \text{min}^{-1}$, respectively) and low K_m (0.329 and 9.23 μM , respectively).

To evaluate the ability of CPA4 to cleave a larger number of peptides, a quantitative peptidomics approach using differen-

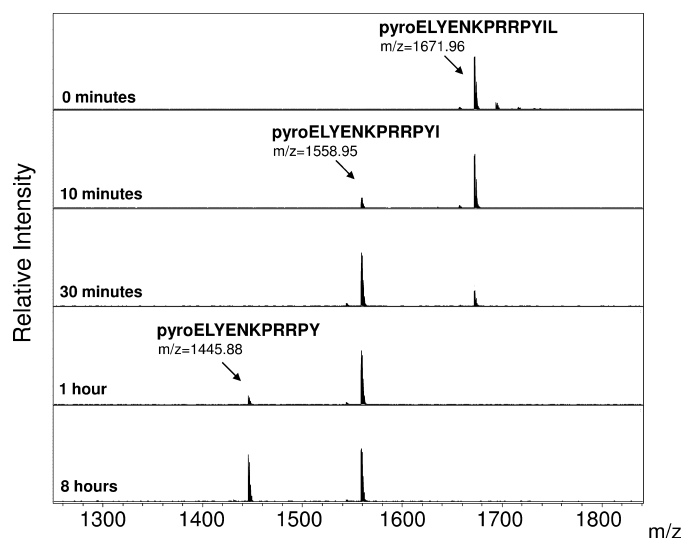


FIGURE 3. MALDI-TOF MS analysis of neurotensin cleavage by CPA4. MALDI-TOF MS spectra of 50 μM neurotensin incubated for different times at 37 °C with 20 nM CPA4. Numbers above the major peaks indicate the monoisotopic masses of the MH⁺ ion (m/z). The peak with a mass of 1671.96 Da corresponds to neurotensin (theoretical monoisotopic mass of neurotensin = 1671.91 Da), and a first cleavage product with a mass of 1558.95 Da formed in the presence of CPA4 corresponds to the peptide lacking the C-terminal Leu (theoretical mass = 1558.84 Da), and a second cleavage product with mass of 1445.88 Da corresponds to the peptide lacking both C-terminal Leu and Ile (theoretical mass = 1445.75 Da).

TABLE 2
Kinetic constants for the hydrolysis of biological peptides by CPA4

Peptide	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$\mu\text{M}^{-1} \cdot \text{min}^{-1}$
Neurotensin	0.240 ± 0.017	0.329 ± 0.049	43.8 ± 9.5
Met-enkephalin-Arg-Phe	1.45 ± 0.03	9.23 ± 0.64	9.43 ± 0.86
Angiotensin I	8.23 ± 0.34	227 ± 25	2.18 ± 0.31
Leu-enkephalin	2.33 ± 0.11	165 ± 19	0.85 ± 0.13
Met-enkephalin	3.43 ± 0.16	550 ± 52	0.374 ± 0.053

tial isotopic tagging and MS was used. For this, peptides were extracted from heat-inactivated mouse brains and purified away from proteins by microfiltration (10-kDa filter cut-off) and then incubated with varying amounts of purified CPA4 or in the absence of enzyme, for 1.5 h (Fig. 4). After heat inactivation of CPA4, the various samples were labeled with one of four isotopic tags, combined, and analyzed by liquid chromatography/mass spectrometry (LC/MS). Over 100 peptides were detected in the LC/MS analysis, including many of the brain peptides tested in supplemental Table S2 (neurotensin, PEN, big SAAS, Met-enkephalin-Arg-Phe (*i.e.* the proenkephalin heptapeptide), little SAAS, Leu-enkephalin, Met-enkephalin, and big LEN). Angiotensin and bradykinin were not detected in the brain peptidome, reflecting the low abundance of these peptides in the brain. Quantification of the relative peptide levels in the enzyme-treated and control incubations was performed by determining peak intensity; representative data are indicated in Fig. 4. Many of the observed peptides showed roughly equal peak intensities; these are neither substrates nor products of CPA4 under the incubation conditions employed. Some peptides showed a decrease in peak intensity only for the sample incubated with the highest amount of enzyme; these are weak substrates of CPA4. Other peptides showed a large decrease in peak intensity for both the highest and the medium amount of

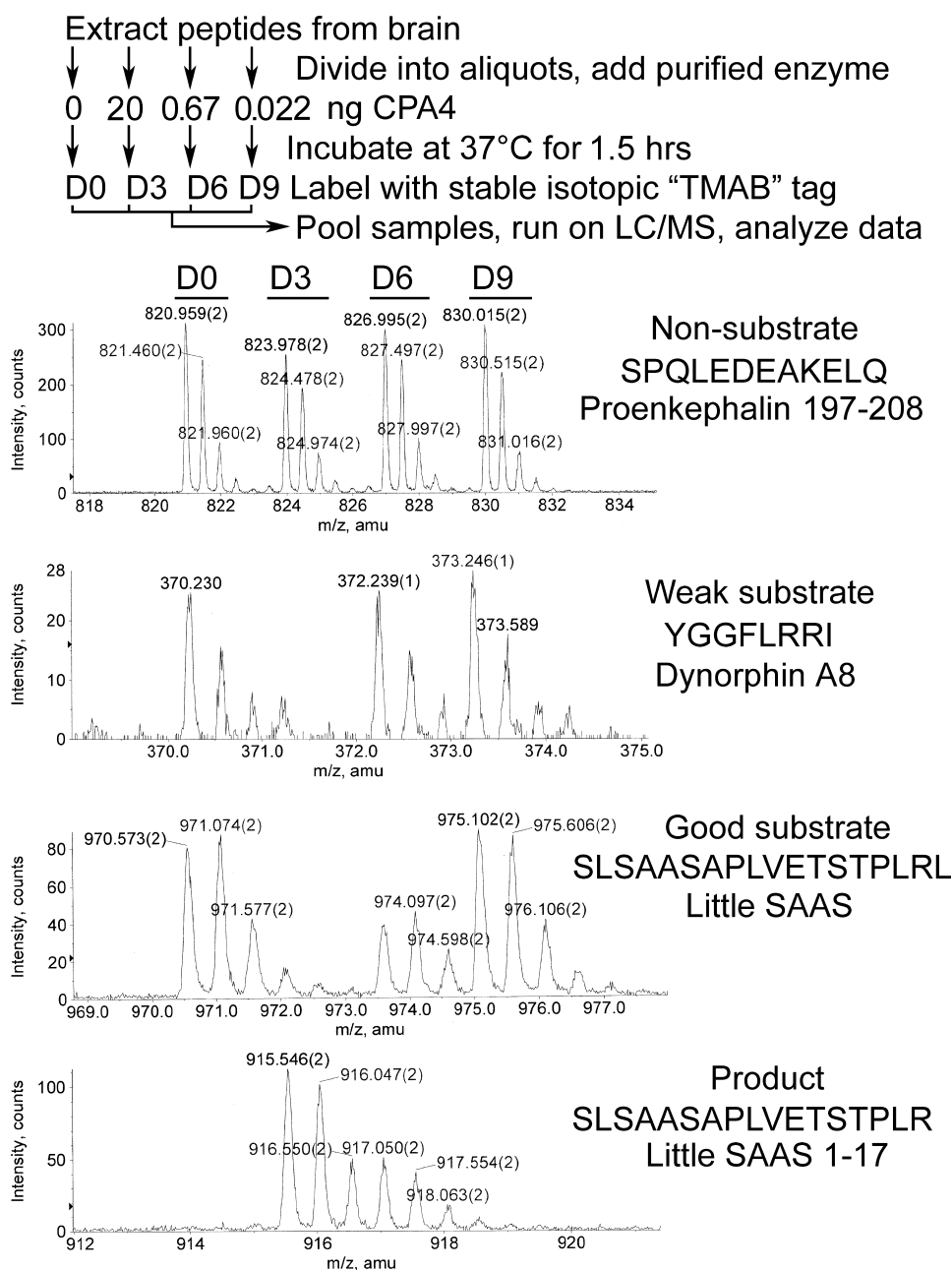


FIGURE 4. Quantitative peptidomics scheme and representative results. Peptides were extracted from microwave-irradiated mouse brain and separated from proteins by microfiltration with a 10-kDa filter. Aliquots were incubated with purified CPA4 (20 ng or 30-fold dilutions) or in the absence of CPA4. After incubation, the samples were labeled with one of four stable isotopic TMAB tags that differ in the number of hydrogens and deuteriums. Samples were then pooled and analyzed by LC/MS. Representative data are shown. Peptides that showed less than a 25% decrease in peak intensity for the sample incubated with the highest concentration of enzyme (the D3 TMAB tag) were considered non-substrates, as shown for the proenkephalin fragment SPQLEDEAKELQ. Peptides that showed a major decrease with the highest concentration of enzyme (>50% decrease) but less than a 25% decrease with the medium concentration of enzyme were considered weak substrates. An example of a weak substrate is the neuropeptide dynorphin A8. Peptides that showed a substantial decrease in peak intensity for the highest concentration of enzyme and at least a 25% decrease for the medium concentration of enzyme were considered good substrates. An example is little SAAS, an 18-residue peptide with a C-terminal Leu. Peptides that showed an increase in peak intensity for the samples incubated with enzyme were considered products of CPA4. An example is little SAAS 1–17, which lacks the C-terminal Leu of little SAAS.

enzyme; these are good substrates of CPA4. Finally, some peptides showed a large increase in peak intensity when incubated with enzyme; these are products of CPA4. Control studies in which aliquots of mouse brain peptides were isotopically tagged and analyzed by LC/MS, without incubation with enzyme,

showed four equal peaks for all of the peptides observed in the present study (19). Approximately half of the detected peptides could be identified by MS/MS sequence analysis. In addition to these sequenced peptides, several other peptides were tentatively identified based on matches to known or predicted peptides; the criteria for these matches included an observed monoisotopic mass that was within 0.004% of the theoretical mass, a correct charge (equal to the number of basic residues plus the N terminus), and a correct number of isotopic tags incorporated (based on the number of free amines in the peptide). These tentatively identified peptides are included in Tables 3–5 and supplemental Table S3 with the sequence surrounded by parentheses; all other sequences in these tables were confirmed by MS/MS sequencing (in addition to fitting the above criteria).

Analysis of the C-terminal residue (*i.e.* the P1' position) of the 16 best substrates of CPA4 identified from the brain peptidome showed a predominance of Leu and Phe, with 14 of the 16 peptides containing one of these two amino acids (Table 3 and Fig. 5). The other two peptides in this group had C termini of Tyr or Val. Similar analysis of the 24 weak substrates of CPA4 showed these 4 amino acids to be present in the C-terminal position, in addition to Ile, Met, Ala, Thr, and His (Table 4 and Fig. 5). Analysis of the products of CPA4 focused on the downstream residue that was removed; of the 10 products that were identified, six required removal of a Leu, and the other four required removal of an Ile, Val, Tyr, or Ala (Table 5). Analysis was also performed on the peptides that were neither substrates nor products of CPA4 (supplemental Table S3 and Fig. 5). Of the 50 peptides in this category, only a small number had C-terminal

Leu or Phe residues; the majority of these peptides contained C-terminal residues that were basic (Lys and Arg), acidic (Asp and Glu), polar (Ser, Asn, and Gln), or otherwise not represented in the best substrate group (Ala, His, and Pro).

Characterization of Human Carboxypeptidase A4

TABLE 3

Good substrates of CPA4

Good substrates are defined as those peptides affected (>25% decrease) by the medium concentration of enzyme and with the high concentration of enzyme greatly reducing the level of peptide (>80% decrease). Sequences were determined by MS/MS analysis, as described under in "Experimental Procedures." Mox, oxidized methionine; pE, pyroGlu; z, charge; T, number of isotopic tags incorporated into peptide; Obs M, observed monoisotopic mass; Theor M, theoretical monoisotopic mass; ppm, difference between Obs M and Theor M in parts per million; Ratio CPA4/no enzyme, the ratio in peak intensity between the sample incubated with enzyme and the sample incubated without enzyme. When the peak intensity was below the background, the ratio is expressed as <0.10 or <0.20, depending on the signal strength and background levels.

Protein name	Peptide name	Sequence	z	T	Obs M	Theor M	ppm	Ratio, CPA4/no enzyme		
								Low	Med	High
Peptidylprolyl isomerase A	118-129	KTEWLDGKHWVF	4	3	1457.74	1457.767	-21	0.81	<0.10	<0.10
Chromogranin A	374-388	LEGEDDPDRSMKLSF	3	2	1737.82	1737.788	17	1.13	<0.10	<0.10
Proenkephalin	Neurotensin	pELYENKPRRPYL	3	1	1671.94	1671.910	18	1.27	<0.20	<0.20
Proenkephalin	Neuromedin N	KIPYIL	2	2	745.47	745.474	1	1.03	0.21	<0.10
Proenkephalin	Heptapeptide	YGGFMRF	2	1	876.41	876.395	12	0.94	0.37	<0.10
Proenkephalin	Heptapeptide, Met-oxidized	YGGF-Mox-RF	2	1	892.40	892.398	8	1.03	0.40	<0.20
Peroxioredoxin V	N-terminus	APIKVGDAIPSVEVF	2	2	1540.87	1540.850	14	0.95	0.40	<0.20
Synaptosomal-associated protein (Snap91)	C-terminus	SPSPTPATQSPKPPAKDPLADLNKDFL	5	5	3072.68	3072.649	10	0.96	0.48	<0.10
ProSAAS	Little SAAS	SLSAASAPLVETSTPLRL	2,3	1	1812.02	1812.007	9	1.04	0.57	<0.10
Chromogranin B	64-86	SGKEVKGEEKGENQNSKFEVRL	5,6	5	2604.38	2604.351	11	1.13	0.66	<0.10
Chromogranin B	600-613	QYDGVAVELDQLLHY	2	1	1662.84	1662.789	28	1.04	0.67	<0.10
Propeptidyl-amidating-monooxygenase	Cleaved pro peptide	FRSPLSVF	2	1	951.53	951.527	-1	1.08	0.67	<0.20
ProSAAS	PEN	SVDQDLGPEVPPENVLGLLRV	2,3	1	2316.27	2316.232	17	1.04	0.71	<0.10
ProSAAS	PEN-20	SVDQDLGPEVPPENVLGLL	2	1	2061.12	2061.063	26	1.53	0.72	<0.20
Elongation factor 1 beta 2	N-terminus	GFGDLKTPAGLQVL	2	2	1414.78	1414.782	2	0.98	0.74	<0.20
ProSAAS	Big SAAS	ARPVKEPRSLSAASAPLVETSTPLRL	5	2	2745.61	2745.550	21	1.14	0.75	<0.10

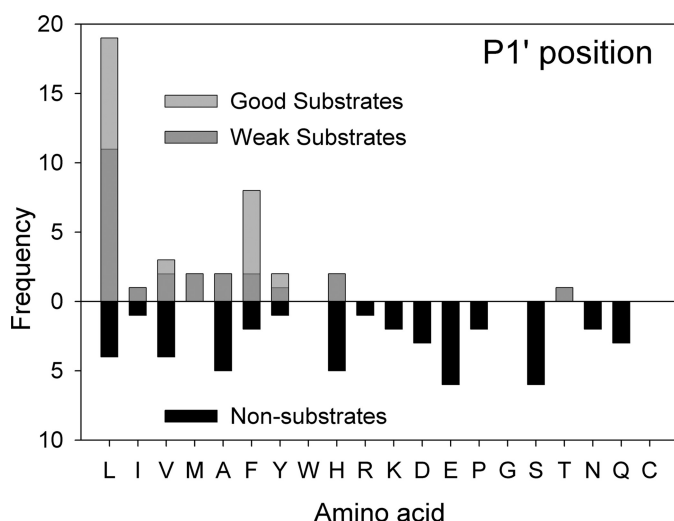


FIGURE 5. Analysis of the C-terminal residue (P1') of peptides detected using the quantitative peptidomics technique. The number of times each amino acid was present in the C-terminal position was determined for good substrates, weak substrates, and non-substrates, as defined in the legend to Fig. 4.

The finding that some but not all of the peptides with C-terminal aliphatic and aromatic residues could be cleaved by CPA4 under the conditions tested suggested that additional residues contributed to the preference of this enzyme for particular peptides. To analyze this, we examined the P1 residue in all peptides with a C-terminal hydrophobic residue. Altogether, there were 62 peptides with a C-terminal Leu, Ile, Val, Met, Ala, Phe, Tyr, His, or Thr; of these, 40 were found to be substrates, and 22 were not cleaved under the conditions tested. The majority of the substrates contained an aliphatic residue (Leu, Ile, Val, Met, or Ala), aromatic (Phe or Tyr), or basic (Arg or Lys) residue in the P1 position (Fig. 6). Furthermore, none of the substrates contained an acidic residue (Asp or Glu), a Pro, or a

Gly in the P1 position. In contrast, only two of the non-cleaved peptides had P1 aliphatic, aromatic, or basic residues (Fig. 6). Furthermore, many of these non-cleaved peptides had acidic or α -helix-breaking residues in the P1 position. Thus, it appears that although the C-terminal residue plays a critical role in determining specificity of CPA4, the P1 residue also contributes to the cleavage specificity.

DISCUSSION

Summary of Cleavage Specificity of CPA4—The major finding of the present study is the cleavage specificity of CPA4, which has not been previously examined. Because of the potential link between CPA4 and some types of cancer (described in the Introduction), it is important to determine the substrate specificity of this enzyme so that predictions can be made regarding its biological role(s). Based on the present results, CPA4 is clearly a CPA-like enzyme with specificity for C-terminal aliphatic and aromatic residues, consistent with the substrate binding pocket predicted from analysis of the crystal structure of CPA4 (14–16). The three types of substrates used in the present study (small chromogenic substrates, selected synthetic peptides, and a mixture of brain peptides) provide complementary information. Although the small chromogenic substrates are ideal for kinetic analysis of k_{cat} and K_m values, these are not natural substrates, and the chromogenic group can potentially affect the cleavage specificity. Synthetic peptides are useful to study precise substrate/product relationships but are limited by the expense and the time to analyze each peptide. The quantitative peptidomics approach provides a great deal of information regarding which peptides are substrates, which are products, and which are not cleaved by CPA4. However, this approach does not provide kinetic information, and it is not possible to follow individual reactions, as can be done with the synthetic peptides. Therefore, these three

TABLE 4
Weak substrates of CPA4

Weak substrates are defined as those peptides that were not substantially affected by the medium concentration of enzyme (less than 25% change) but showed a decrease with the high concentration of enzyme. Abbreviations are as defined in Table 3. Peptide sequences in parentheses are tentative identifications, based on similarities to known peptides, without MS/MS sequence information. These similarities include the monoisotopic mass, the charge (based on the number of basic residues in the peptide), and the number of isotopic tags incorporated into the peptide (based on the number of free amines). See "Experimental Procedures" for further details.

Protein name	Peptide name	Sequence	z	T	Obs M	Theor M	ppm	Ratio, CPA4/no enzyme		
								Low	Med	High
Provasopressin	151-end	VQLAGTRESVDSAKPRVY	4	2	1975.08	1975.049	15	0.66	0.80	<0.10
Actin (beta or gamma)	169-177 of beta	ALPHAILRL	3	1	1002.64	1002.641	0	1.15	0.82	<0.10
Cathepsin D	138-155	YTVFDRDNNRVGFANAVV	3	1	2056.07	2056.013	26	0.91	0.83	<0.10
Secretogranin II	287-316	(SGQLGLPDEENRRESKQDLSKEDASKVITYL)	5	3	3376.71	3376.675	10	0.91	0.85	<0.10
Peptidylprolyl isomerase A	26-39	ADKVPKTAENFRAL	4	3	1558.86	1558.847	7	1.11	0.92	0.21
Proteasome subunit, beta type, 6	34-41	(TTIMAVQF)	2	1	909.47	909.463	8	0.97	0.92	<0.20
Thioredoxin 1	N-terminus	VKLIESKEAFQEAL	3	3	1603.91	1603.882	15	1.01	0.93	0.38
Vacuolar ATP synthase subunit 2	C-terminus	EVRPQVHPNYRVTV	4	1	1692.91	1692.913	-2	0.87	0.93	<0.10
Secretogranin II	300-316	ESKDQDSEDASKVITYL	3	3	1924.98	1924.960	13	1.09	0.95	<0.10
Prodynorphin	Dynorphin A8	YGGFLRR	3	1	980.56	980.556	-1	0.97	0.96	<0.10
Ribosomal protein S21	C-terminus	AKADGIVSKNF	3	3	1148.63	1148.619	7	0.90	0.99	<0.10
Proenkephalin	Octapeptide	YGGFMRS	2	1	929.45	929.451	-3	1.03	1.00	0.17
Triose-phosphate isomerase	149-163	KVIADNVKDWSKVVL	4	4	1712.99	1712.982	5	0.92	1.00	<0.10
Chromogranin A	392-402	AYGFRDPGPQL	2	1	1219.61	1219.600	12	1.02	1.01	0.45
Proenkephalin	Leu-Enkephalin	YGGFL	1	1	555.27	555.269	10	1.10	1.01	<0.10
Clathrin light chain A	C-terminus	SVLISLKKQAPLVH	3	2	1403.85	1403.850	0	1.08	1.02	<0.12
Chromogranin A	374-390	LEGEDDPPDRSMKLSFRT	4	2	1994.97	1994.937	16	1.06	1.03	<0.10
Voltage-dependent anion channel protein 1	C-terminus	AGGHKLGLGLEFQA	3	2	1396.76	1396.746	10	1.08	1.03	0.23
Peptidylprolyl isomerase A	23-39	ELFADKVPKTAENFRAL	4	3	1948.06	1948.042	10	1.00	1.04	<0.10
Peptidylprolyl isomerase A	84-92	EDENFILKH	3	2	1143.56	1143.556	6	1.26	1.08	<0.20
Peptidylprolyl isomerase A	84-100	EDENFILKHTGPGILSM	3	2	1899.96	1899.948	5	1.10	1.10	<0.20
Procholecystokinin	46-63	AVLRTDGEPRARLGALLA	4	1	1878.11	1878.080	14	1.07	1.13	<0.10
Procholecystokinin	46-62	AVLRTDGEPRARLGALL	4	1	1807.05	1807.043	3	1.19	1.16	<0.10
Proenkephalin	Met-Enkephalin	YGGFM	1	1	573.23	573.226	10	1.15	1.20	0.18

TABLE 5
Products of CPA4

These peptides are defined as those that increased >100% with one or more of the concentrations of CPA4. Abbreviations are as defined in Table 3. nd, not detected. For those peptides not detected in the "no enzyme" control, the ratio is expressed using the greater than sign; relative levels between the different concentrations of enzyme are reflected in the different lower limits for the increase. The amino acid cleaved by CPA4 to generate the observed peptide is indicated in column 4.

Protein name	Peptide name	Sequence	Cleaved	z	T	Obs M	Theor M	ppm	Ratio, CPA4/no enzyme		
									Low	Med	High
Procholecystokinin	43-60	(QLRAVLRDGEPRARLGA)	L	5	1	1978.14	1978.118	13	1.37	2.33	1.52
Procholecystokinin	46-60	AVLRTDGEPRARLGA	L	4	1	1580.88	1580.875	6	1.45	1.64	5.05
Procholecystokinin	46-61	AVLRTDGEPRARLGA	L	4	1	1693.97	1693.970	2	1.00	3.60	12
Proneurotensin	Neurotensin 1-12	pELYENKPRRPYI	L	3	1	1558.86	1558.836	15	>5.6	>19	>7.2
Proneurotensin	Neurotensin 1-11	pELYENKPRRPY	I	3	1	1445.77	1445.752	12	nd	nd	>10
ProSAAS	Little SAAS 1-17	SLSAASAPLVETSTPLR	L	2	1	1698.94	1698.915	16	nd	>6.6	>11
ProSAAS	PEN 1-21	SVDQDLGPEVPPENVLGALLR	V	2,3	1	2217.19	2217.164	13	>1.4	>9.7	>20
ProSAAS	Big SAAS 1-25	ARPVKPRSLSAASAPLVETSTPLR	L	5	2	2632.50	2632.466	13	nd	>8.8	>19
Chromogranin B	600-612	QYDGAELDQLLH	Y	2	1	1499.75	1499.726	14	1.00	>10	>10
Chromogranin B	438-453	LLDEGHYPVRESPIDT	A	3	1	1839.92	1839.900	10	0.94	1.09	2.14

approaches have overlapping strengths and collectively provide a more complete picture than a single approach.

Taken together, the optimal C-terminal residue for CPA4 cleavage is Leu, Ile, Val, Met, Tyr, or Phe (Fig. 7). Other acceptable amino acids in this position include Ala, His, Thr, or Trp. CPA4 does not cleave C-terminal Pro, basic residues (Arg and Lys), acidic residues (Asp and Glu), or polar residues, such as Ser, Asn, or Gln. Although Thr can be considered a polar residue, it is less polar than Ser. No information on Gly or Cys in the C-terminal position is available. The P1 position also has an influence on cleavage specificity, with aliphatic, aromatic, and basic residues favorable in this position (Fig. 7). Certain amino acids have a negative influence in the P1 position, such as acidic residues, Pro, and Gly (Fig. 7). No information on Trp or Cys is available for the P1 position.

Comparison among CPAs—The FA-Phe-Xaa series of substrates offer higher k_{cat}/K_m values than other small synthetic

substrates, allowing measurements of CPA2 toward substrates with small hydrophobic C-terminal amino acids, which have not previously been reported. In most cases, the improvement of catalytic efficiency for CPA2 toward FA-Phe-Xaa substrates versus N-benzyloxycarbonyl-Gly_n-Xaa substrates is due to lower K_m values, but in some cases an increase in k_{cat} values also contributes to the difference (33). This effect may be explained by the different amino acids in the penultimate position of these substrates (Phe versus Gly); if CPA2 is similar to CPA4 as described above from the peptidomics analysis, Phe is much preferred over Gly in the P1 position. In addition, the FA-blocking group is known to enhance the CP activity (31). The kinetic data for CPA2 fully agree with the data reported by Reverter *et al.* (33), showing a preference for FA-Phe-Trp and FA-Phe-Phe. Compared with FA-Phe-Trp, the k_{cat}/K_m values are 85- and 335-fold lower for FA-Phe-Leu and FA-Phe-Ile, respectively. In contrast with these previous reports, we were able to estimate

Characterization of Human Carboxypeptidase A4

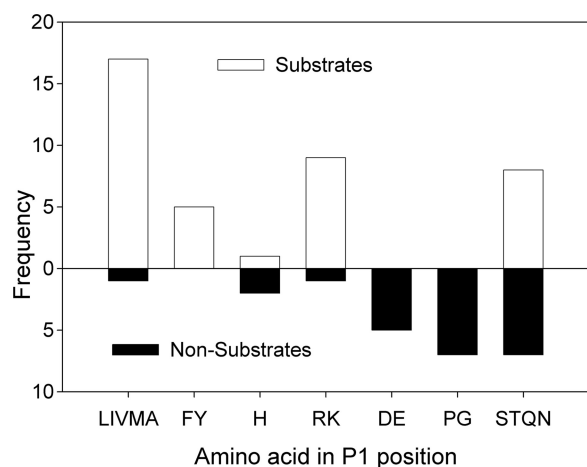


FIGURE 6. Analysis of the P1 residue of peptide containing hydrophobic C-terminal residues. For this analysis, only peptides that were substrates or potential substrates were considered, based on the analysis shown in Fig. 5. Those peptides with acidic, basic, or polar C-terminal residues were excluded. This analysis included 40 substrates (good and weak substrates were combined) and 22 non-substrates with similar C-terminal residues as the substrates (*i.e.* Leu, Ile, Val, Met, Ala, Phe, Tyr, and His). The frequency of the various amino acids in the P1 position of each group were determined and combined into groups based on amino acid type: aliphatic (Leu, Ile, Val, Met, and Ala), aromatic (Phe and Tyr), basic (Arg and Lys), acidic (Asp and Glu), α -helix breakers (Pro and Gly), and polar (Ser, Thr, Gln, and Asn). His was placed into its own group, and there were no data for Cys or Trp because these amino acids were not present in the P1 position in any of the peptides analyzed.

Optimal	LIVM	LIVMYF
	FYRK	
Acceptable	AHSTQN	AHTW
	- P1 - P1'	
Inhibitory	DEPG	RKDE PSNQ

FIGURE 7. Summary of cleavage specificity of CPA4, based on results from synthetic chromogenic substrates, synthetic peptides, and endogenous mouse brain peptides. Those amino acids considered most favorable for cleavage by CPA4 are listed in the *top row* and include Leu, Ile, Val, Met, Tyr, and Phe in the P1' position and these same residues plus Arg and Lys in the P1 position. Residues that are acceptable in these positions are listed in the *middle row*, and those residues that appear to be inhibitory for cleavage are listed in the *bottom row*.

k_{cat}/K_m values for FA-Phe-Met and FA-Phe-Val that are ~ 1000 times lower than the value for FA-Phe-Trp.

Although CPA1 has been well studied over the past 50 years, particularly bovine CPA1 (originally referred to as CPA in the literature), the kinetic constants for human CPA1 have not been previously reported. The preference of CPA1 for Phe in the C-terminal position is more marked than that of CPA4 when the k_{cat}/K_m values for different substrates are compared. The k_{cat}/K_m ratio of CPA1 for FA-Phe-Phe is ~ 2 -fold greater than that of FA-Phe-Leu and FA-Phe-Val, 4 times higher than that of FA-Phe-Ile, and 10-fold higher than the corresponding values for FA-Phe-Trp, FA-Phe-Met, and FA-Phe-Ala. When k_{cat}/K_m values are compared among the three enzymes, CPA1 and CPA2 appear to be more efficient than CPA4 in the removal of C-terminal residues from peptides. For example,

k_{cat}/K_m values for FA-Phe-Phe are approximately the same for CPA1 and CPA2 and 4 times lower for CPA4.

A comparison between CPA4 and CPA6 activities toward the same group of peptides shows several differences. CPA6 cleaves both Leu and His from the C terminus of angiotensin I and des-Asp¹ angiotensin I, whereas CPA4 only removes the Leu and not the His under the conditions described in [supplemental Table S2](#). However, CPA4 can cleave both the Leu and His in angiotensin when higher enzyme and peptide concentrations are used (data not shown). Furthermore, FA-Phe-His is cleaved by CPA4 at a low rate, and in the peptidomics study, two peptides that contain C-terminal His residues were found to be weak substrates of CPA4. Therefore, CPA4 is able to cleave His from the C terminus of some substrates. In addition to CPA4 and CPA6, previously it was found that CPA1 (34) and CPA3 (35) can cleave C-terminal His residues. Although His is commonly considered to be a basic residue, at neutral pH values, it will be mainly unprotonated and resemble an aromatic/aliphatic residue. Thus, it is not surprising that this residue is cleaved by CPA-like enzymes.

The substrate specificity of CPA4 determined in the present study fits very well with predictions made from structural analysis of this enzyme in complex with a hexapeptide (16) and with results from structural analysis of many CPAs and CPBs (1). The specificity of CPA4 for C-terminal hydrophobic residues, such as Leu, Ile, Val, Met, Tyr, and Phe, and weaker hydrolysis of Trp match more closely the specificity of CPA1 rather than CPA2. The S1' specificity pocket is delimited by the side chains in positions 194, 203, 207, 243, 247, 250, 253–255, and 268 (using the numbering system of bovine CPA1). The residue in position 255 (Ile for CPA4) is considered to be the major determinant of substrate specificity for carboxypeptidases of the CPA/CPB subfamily (36). In CPA4, the residues in positions 203, 247, and 250 (numbering system of CPA1), which are Met, Val, and Ala, respectively, also contribute to the hydrophobicity of the S1' binding pocket. A comparison of the amino acids that shape the S1' pocket between CPA1, CPA2, and CPA4 explains the strong preference of hCPA2 for bulkier hydrophobic amino acids in comparison with CPA1 and CPA4 ([supplemental Table S4 and Fig. S1](#)). The presence of Ala in position 268 in CPA2, in contrast to the Thr present in CPA1 and CPA4, makes the specificity pocket of CPA2 larger and more hydrophobic, explaining the specificity of CPA2 for large amino acids like Trp (37, 38). The amino acid in position 253 may also be responsible for this difference. The preference of CPA1 for C-terminal Phe is more marked than that of CPA4; this preference can be explained by the presence of a more hydrophobic environment in the S1' pocket of CPA1 provided by Ile²⁴³ and Ile²⁴⁷, compared with the Thr and Val in similar positions in CPA4.

In the present study, the S1 subsite was also found to contribute to the specificity of CPA4 (Fig. 7); critical residues in this subsite are the Tyr in position 198 and Ser in position 199 (16). Other residues known to contribute to the shape of the S1 subsite of CPAs include Val²⁴⁷, Tyr²⁴⁸, Phe²⁷⁹, and Glu²⁷⁰, which form a mainly hydrophobic pocket that may explain the preference of CPA4 for substrates with hydrophobic amino acids in the P1 position. The preference of CPA4 for positively charged amino acids over negatively charged amino acids in the P1 posi-

tion could be explained by interactions between the side chain of the P1 residue with the polar portion of Tyr¹⁹⁸ and Ser¹⁹⁹ in the S1 binding site (1).

Potential Function of CPA4—The finding that PCPA4 is secreted from cells in a soluble form and, once activated, has a neutral pH optimum suggests a function in the extracellular environment rather than within the secretory pathway. Although trypsin was used to activate PCPA4 in the present study, this is not likely to represent the physiological activating enzyme because trypsin is pancreatic, whereas CPA4 has a broader distribution. There are a number of trypsin-like enzymes expressed in the brain and other tissues where PCPA4 is found (39). Based on modeling and structural considerations, the conversion of PCPA4 into active CPA4 requires cleavage at a single Arg in the sequence EGQER-SS. It is possible that the activation of PCPA4 may be an important control point that regulates the active form of the enzyme.

The distribution of CPA4 provides important clues as to the function of this enzyme. Huang *et al.* (9) found CPA4 mRNA to be expressed at high levels in different prostate cancer cell lines and at extremely low levels in some normal human tissues, including prostate and pancreas. CPA4 mRNA was induced in prostate cancer cell lines upon treatment with 10 mM sodium butyrate, indicating that its expression is associated with *in vitro* differentiation. It was also reported that CPA4 expression is associated with hormone-regulated tissues. Through an imprinting analysis, Kayashima *et al.* (12) found that CPA4 mRNA was also highly expressed in fetal tissues that are growing or differentiating with the transcriptionally active state of chromatin. Using RT-PCR, Bentley *et al.* (11) detected CPA4 expression in a wide variety of tissues, including placenta, testis, uterus, heart, brain, intestine, kidney, and muscle. In mouse brain, *in situ* hybridization showed relatively high expression of CPA4 mRNA in the olfactory bulb, specifically in the granular and mitral layers (40), in an expression pattern similar to that of CPA6 mRNA (41). A United States patent using TaqMan analysis of human tissues reported expression of CPA4 in kidney, spinal cord, and brain, with moderate levels in cortex and considerably higher levels in hypothalamus, especially in the arcuate and paraventricular nuclei (42). Taken together, it is clear that CPA4 mRNA is present in many tissues.

The association of CPA4 mRNA levels with differentiation and/or cancerous cells and tissues raises the possibility that this enzyme plays an important role in cleaving a factor or factors that are critical to the differentiated state. Many secreted factors are known to function as growth factors or to otherwise enable tissues to grow and metastasize (such as secreted proteases), and CPA4 may contribute to these processes. Some of the peptides found to be substrates in the present study include neurotensin, granins like chromogranin A and B and secretogranin II, and opioid peptides such as Met- and Leu-enkephalin. The cleavage performed by CPA4 would eliminate the binding of these peptides to their receptors. Although enkephalin is a well known neuropeptide that functions in analgesia and the reward pathway, additional roles for enkephalin include synaptogenesis and neurite outgrowth (43, 44). Some granin-derived peptides have been postulated to participate as regulators of tissue repair, inflammatory reaction, and innate immunity (45).

Additional roles for some of these products include the stimulation of proliferation in tumor cells (46) and invasive potential of prostate cancer cells partially through enhancement of cell mobility (47). Similarly, neurotensin is also a well studied neuropeptide that has recently been proposed to contribute to the progression of several types of cancer, including prostate, breast, and pancreatic cancer (48–50). Because the enkephalins, secretogranins, and neurotensin are secreted from some of the same tissues where CPA4 is also secreted, it is likely that this enzyme plays a role in the termination of the action of these peptides as well as many others. A role for CPA4 in the inactivation of peptides that function in cell proliferation would potentially provide a mechanism to account for the previous studies linking CPA4 with aggressiveness of some forms of cancer. Additional research efforts are needed to characterize and understand the biological effects of this enzyme and its single-nucleotide polymorphism variant(s) in the tumoral process.

Acknowledgments—Mass spectrometry was performed in the Laboratory for Macromolecular Analysis and Proteomics of the Albert Einstein College of Medicine, directed by Dr. Ruth Angeletti. We acknowledge the technical assistance of the Cell Culture Facility of the Institut de Biologia i de Biomedicina and the Microscopy Facility, both at the Universitat Autònoma de Barcelona. We thank Dr. Peter Lyons for helpful comments.

REFERENCES

- Gomis-Rüth, F. X. (2008) *Crit. Rev. Biochem. Mol. Biol.* **43**, 319–345
- Rawlings, N. D., Morton, F. R., and Barrett, A. J. (2006) *Nucleic Acids Res.* **34**, D270–D272
- Kalinina, E., Biswas, R., Berezniuk, I., Hermoso, A., Aviles, F. X., and Fricker, L. D. (2007) *FASEB J.* **21**, 836–850
- Rodriguez de la Vega, M., Sevilla, R. G., Hermoso, A., Lorenzo, J., Tanco, S., Diez, A., Fricker, L. D., Bautista, J. M., and Avilés, F. X. (2007) *FASEB J.* **21**, 851–865
- Pejler, G., Abrink, M., Ringvall, M., and Wernersson, S. (2007) *Adv. Immunol.* **95**, 167–255
- Lyons, P. J., Callaway, M. B., and Fricker, L. D. (2008) *J. Biol. Chem.* **283**, 7054–7063
- Leung, L. L., Myles, T., Nishimura, T., Song, J. J., and Robinson, W. H. (2008) *Mol. Immunol.* **45**, 4080–4083
- Arolas, J. L., Vendrell, J., Aviles, F. X., and Fricker, L. D. (2007) *Curr. Pharm. Des.* **13**, 349–366
- Huang, H., Reed, C. P., Zhang, J. S., Shridhar, V., Wang, L., and Smith, D. I. (1999) *Cancer Res.* **59**, 2981–2988
- Witte, J. S., Goddard, K. A., Conti, D. V., Elston, R. C., Lin, J., Suarez, B. K., Broman, K. W., Burmester, J. K., Weber, J. L., and Catalona, W. J. (2000) *Am. J. Hum. Genet.* **67**, 92–99
- Bentley, L., Nakabayashi, K., Monk, D., Beechey, C., Peters, J., Birjandi, Z., Khayat, F. E., Patel, M., Preece, M. A., Stanier, P., Scherer, S. W., and Moore, G. E. (2003) *J. Med. Genet.* **40**, 249–256
- Kayashima, T., Yamasaki, K., Yamada, T., Sakai, H., Miwa, N., Ohta, T., Yoshiura, K., Matsumoto, N., Nakane, Y., Kanetake, H., Ishino, F., Niikawa, N., and Kishino, T. (2003) *Hum. Genet.* **112**, 220–226
- Ross, P. L., Cheng, I., Liu, X., Cicek, M. S., Carroll, P. R., Casey, G., and Witte, J. S. (2009) *BMC Cancer* **9**, 69
- García-Castellanos, R., Bonet-Figueroa, R., Pallarés, I., Ventura, S., Avilés, F. X., Vendrell, J., and Gomis-Rüth, F. X. (2005) *Cell Mol. Life Sci.* **62**, 1996–2014
- Pallarés, I., Bonet, R., García-Castellanos, R., Ventura, S., Avilés, F. X., Vendrell, J., and Gomis-Rüth, F. X. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 3978–3983
- Bayés, A., Fernández, D., Solà, M., Marrero, A., García-Piqué, S., Avilés,

Characterization of Human Carboxypeptidase A4

- F. X., Vendrell, J., and Gomis-Rüth, F. X. (2007) *Biochemistry* **46**, 6921–6930
17. Blumberg, S., and Vallee, B. L. (1975) *Biochemistry* **14**, 2410–2419
 18. Bieth, J. G. (1995) *Methods Enzymol.* **248**, 59–84
 19. Morano, C., Zhang, X., and Fricker, L. D. (2008) *Anal. Chem.* **80**, 9298–9309
 20. Che, F. Y., Zhang, X., Berezniuk, I., Callaway, M., Lim, J., and Fricker, L. D. (2007) *J. Proteome Res.* **6**, 4667–4676
 21. Che, F. Y., Lim, J., Pan, H., Biswas, R., and Fricker, L. D. (2005) *Mol. Cell Proteomics* **4**, 1391–1405
 22. Zhang, X., Che, F. Y., Berezniuk, I., Sonmez, K., Toll, L., and Fricker, L. D. (2008) *J. Neurochem.* **107**, 1596–1613
 23. Hamstra, D. A., and Rehemtulla, A. (1999) *Hum. Gene Ther.* **10**, 235–248
 24. Novikova, E. G., Reznik, S. E., Varlamov, O., and Fricker, L. D. (2000) *J. Biol. Chem.* **275**, 4865–4870
 25. Byers, L. D., and Wolfenden, R. (1973) *Biochemistry* **12**, 2070–2078
 26. Felber, J. P., Coombs, T. L., and Vallee, B. L. (1962) *Biochemistry* **1**, 231–238
 27. Ryan, C. A., Hass, G. M., and Kuhn, R. W. (1974) *J. Biol. Chem.* **249**, 5495–5499
 28. Reverter, D., Vendrell, J., Canals, F., Horstmann, J., Avilés, F. X., Fritz, H., and Sommerhoff, C. P. (1998) *J. Biol. Chem.* **273**, 32927–32933
 29. Arolas, J. L., Lorenzo, J., Rovira, A., Castellà, J., Avilés, F. X., and Sommerhoff, C. P. (2005) *J. Biol. Chem.* **280**, 3441–3448
 30. Sanglas, L., Avilés, F. X., Huber, R., Gomis-Rüth, F. X., and Arolas, J. L. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 1743–1747
 31. Peterson, L. M., Holmquist, B., and Bethune, J. L. (1982) *Anal. Biochem.* **125**, 420–426
 32. Pallarès, I., Fernández, D., Comellas-Bigler, M., Fernández-Recio, J., Ventura, S., Avilés, F. X., Bode, W., and Vendrell, J. (2008) *Acta Crystallogr. D Biol. Crystallogr.* **D64**, 784–791
 33. Reverter, D., García-Sáez, I., Catasús, L., Vendrell, J., Coll, M., and Avilés, F. X. (1997) *FEBS Lett.* **420**, 7–10
 34. Garcia, K. C., Tallquist, M. D., Pease, L. R., Brunmark, A., Scott, C. A., Degano, M., Stura, E. A., Peterson, P. A., Wilson, I. A., and Teyton, L. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13838–13843
 35. Lundquist, A., Tchougounova, E., Abrink, M., and Pejler, G. (2004) *J. Biol. Chem.* **279**, 32339–32344
 36. Grishin, A. M., Akparov, V. K., and Chestukhina, G. G. (2008) *Protein Eng. Des. Sel.* **21**, 545–551
 37. Faming, Z., Kobe, B., Stewart, C. B., Rutter, W. J., and Goldsmith, E. J. (1991) *J. Biol. Chem.* **266**, 24606–24612
 38. García-Sáez, I., Reverter, D., Vendrell, J., Avilés, F. X., and Coll, M. (1997) *EMBO J.* **16**, 6906–6913
 39. Wang, Y., Luo, W., and Reiser, G. (2008) *Cell Mol. Life Sci.* **65**, 237–252
 40. Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A. F., Boguski, M. S., Brockway, K. S., Byrnes, E. J., Chen, L., Chen, L., Chen, T. M., Chin, M. C., Chong, J., Crook, B. E., Czaplinska, A., Dang, C. N., Datta, S., Dee, N. R., Desaki, A. L., Desta, T., Diep, E., Dolbeare, T. A., Donelan, M. J., Dong, H. W., Dougherty, J. G., Duncan, B. J., Ebbert, A. J., Eichele, G., Estin, L. K., Faber, C., Facer, B. A., Fields, R., Fischer, S. R., Fliss, T. P., Frensley, C., Gates, S. N., Glattfelder, K. J., Halverson, K. R., Hart, M. R., Hohmann, J. G., Howell, M. P., Jeung, D. P., Johnson, R. A., Karr, P. T., Kawal, R., Kidney, J. M., Knapik, R. H., Kuan, C. L., Lake, J. H., Laramee, A. R., Larsen, K. D., Lau, C., Lemon, T. A., Liang, A. J., Liu, Y., Luong, L. T., Michaels, J., Morgan, J. J., Morgan, R. J., Mortrud, M. T., Mosqueda, N. F., Ng, L. L., Ng, R., Orta, G. J., Overly, C. C., Pak, T. H., Parry, S. E., Pathak, S. D., Pearson, O. C., Puchalski, R. B., Riley, Z. L., Rickett, H. R., Rowland, S. A., Royall, J. J., Ruiz, M. J., Sarno, N. R., Schaffnit, K., Shapovalova, N. V., Svisay, T., Slaughterbeck, C. R., Smith, S. C., Smith, K. A., Smith, B. I., Sodt, A. J., Stewart, N. N., Stumpf, K. R., Sunkin, S. M., Sutram, M., Tam, A., Teemer, C. D., Thaller, C., Thompson, C. L., Varnam, L. R., Visel, A., Whitlock, R. M., Wohnoutka, P. E., Wolkey, C. K., Wong, V. Y., Wood, M., Yaylaoglu, M. B., Young, R. C., Youngstrom, B. L., Yuan, X. F., Zhang, B., Zwingman, T. A., and Jones, A. R. (2007) *Nature* **445**, 168–176
 41. Fontenele-Neto, J. D., Kalinina, E., Feng, Y., and Fricker, L. D. (2005) *Brain Res. Mol. Brain Res.* **137**, 132–142
 42. White, D. (April 22, 2004) U. S. Patent 2004/0077001 A1
 43. Davila-García, M. I., and Azmitia, E. C. (1990) *Adv. Exp. Med. Biol.* **265**, 75–92
 44. Escobar, M. L., Barea-Rodríguez, E. J., Derrick, B. E., Reyes, J. A., and Martínez, J. L., Jr. (1997) *Brain Res.* **751**, 330–335
 45. Helle, K. B., Corti, A., Metz-Boutigue, M. H., and Tota, B. (2007) *Cell Mol. Life Sci.* **64**, 2863–2886
 46. Mosca, A., Berruti, A., Russo, L., Torta, M., and Dogliotti, L. (2005) *J. Endocrinol. Invest.* **28**, 141–145
 47. Nagakawa, O., Ogasawara, M., Murata, J., Fuse, H., and Saiki, I. (2001) *Int. J. Urol.* **8**, 65–70
 48. Dupouy, S., Viardot-Foucault, V., Alifano, M., Souazé, F., Plu-Bureau, G., Chaouat, M., Lavaur, A., Hugol, D., Gespach, C., Gompel, A., and Forgez, P. (2009) *PLoS One* **4**, e4223
 49. Myers, R. M., Shearman, J. W., Kitching, M. O., Ramos-Montoya, A., Neal, D. E., and Ley, S. V. (2009) *ACS Chem. Biol.* **4**, 503–525
 50. Vias, M., Burt, G., Culig, Z., Veerakumarasivam, A., Neal, D. E., and Mills, I. G. (2007) *Prostate* **67**, 190–202