Characterization of Carboxypeptidase A6, an Extracellular Matrix Peptidase*

Received for publication, September 12, 2007, and in revised form, December 21, 2007 Published, JBC Papers in Press, January 4, 2008, DOI 10.1074/jbc.M707680200

Peter J. Lyons, Myrasol B. Callaway, and Lloyd D. Fricker

From the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Carboxypeptidase A6 (CPA6) is a member of the M14 metallo-carboxypeptidase family that is highly expressed in the adult mouse olfactory bulb and broadly expressed in embryonic brain and other tissues. A disruption in the human CPA6 gene is linked to Duane syndrome, a defect in the abducens nerve/lateral rectus muscle connection. In this study the cellular distribution, processing, and substrate specificity of human CPA6 were investigated. The 50-kDa pro-CPA6 is routed through the constitutive secretory pathway, processed by furin or a furin-like enzyme into the 37-kDa active form, and secreted into the extracellular matrix. CPA6 cleaves the C-terminal residue from a range of substrates, including small synthetic substrates, larger peptides, and proteins. CPA6 has a preference for large hydrophobic C-terminal amino acids as well as histidine. Peptides with a pentulinate glycine or proline are very poorly cleaved. Several neuropeptides were found to be processed by CPA6, including Met- and Leu-enkephalin, angiotensin I, and neurotensin. Whereas CPA6 converts enkephalin and neurotensin into forms known to be inactive toward their receptors, CPA6 converts inactive angiotensin I into the biologically active angiotensin II. Taken together, these data suggest a role for CPA6 in the regulation of neuropeptides in the extracellular environment within the olfactory bulb and other parts of the brain.

Carboxypeptidases (CP)2 perform many functions in mammals and other species through the cleavage of specific C-terminal amino acids (1, 2). The M14 family of mammalian metallo-CPs is divided into three subfamilies based on amino acid sequence homology, structure, and function (1). These subfamilies are referred to by the names of the first two members of each group that were discovered: the A/B group, the N/E group, and the Nna1/CCP group (3). All metallo-CPs have structurally similar CP domains. In addition to the CP domain, all A/B members contain an N-terminal propeptide that is necessary for folding and is cleaved off upon enzyme activation (1, 4). The majority of A/B and N/E enzymes are secreted and function outside of the cell. Some of the N/E subfamily CPs function in the secretory pathway; examples include CPE and CPD that process hormones and neuropeptides as they pass through the secretory pathway (5–7). CPE is mutated in fat/fat mice, leading to obesity and various neurological deficits (8). Recently a family of cytosolic carboxypeptidases, the CCPs, has been identified (3, 9). The functions of these enzymes have not been well characterized yet, although the first identified member, Nna1/CCP1, is mutated in a classical mouse mutant, the Purkinje cell degeneration mice (10, 11).

The A/B subfamily of metallo-CPs consists of 9 members (1). Three of these enzymes, CPA1, CPA2, and CPB1, are produced by the pancreas and are involved in the digestion of food through the cleavage of C-terminal aliphatic/aromatic (CPA) and basic (CPB) amino acids. Mast cell CPA (also known as the CPA3 gene product) is found in the secretory granules of mast cells and functions in inflammatory processes. CPB2 (also known as CPU, CPR, and TAFI) has an important regulatory role in fibrinolysis (12). Little has been published about CPA4, CPA5, and CPO (13, 14). (CPA4 was originally referred to as CPA3 in Ref. 14 but has been recently renamed by a gene nomenclature committee.) CPA6 was discovered in 2002 in a bioinformatics screen of the human genome (13). Modeling suggested that CPA6 is a CPA-like enzyme that cleaves C-terminal hydrophobic residues (13). Soon after this initial report was published, a human mutation of CPA6 was described (15). This report described a man with a de novo reciprocal balanced translocation resulting in the interruption of the coding sequence for CPA6 on one copy of chromosome 8 at arm 8q13 (15). The patient presented with Duane syndrome, a congenital eye-movement disorder in which eye abduction is restricted or absent and adduction is restricted (16). Two additional patients with Duane syndrome and de novo deletions/translocations of chromosome arm 8q13 have been reported, further supporting a link between CPA6 mutations and Duane syndrome (17, 18). Duane syndrome is generally believed to be caused by a failure of cranial nerve VI to innervate the lateral rectus muscle and the abberant innervation of this muscle by cranial nerve III (19).

The mouse homolog of CPA6 has been cloned and sequenced (20). The distribution of mouse CPA6 mRNA was examined in adult tissues by reverse transcription-PCR and in both adult brain and embryonic tissue by in situ hybridization.
Carboxypeptidase Assays—For all assays, HEK293T cells were plated in 6-well plates and transfected after 24 h with either CPA6 expression plasmid or with pcDNA3.1 alone. Cells extract and medium were run on a 10% SDS-polyacrylamide gel (Bio-Rad) and proteins transferred to nitrocellulose. Western blotting was performed with mouse anti-HA (clone HA-7, Sigma, 1:3000 dilution) and mouse anti-TetraHis (Qiagen, 1:2000 dilution) primary antibodies and IRdye800-conjugated anti-mouse secondary antibodies (Rockland, 1:3000 dilution). Images were obtained by scanning with a LiCor Odyssey. Band quantification was performed with ImageJ software.

Pulse-Chase Analysis—HEK293T cells were transfected in 60-mm plates and grown for 24 h. Cells were washed twice with PBS and incubated in 1 ml of DMEM containing 5% dialyzed PBS and lacking l-methionine and l-cystine. After 30 min, 50 µl of Tran35S-label (MP Biomedicals) corresponding to ~500 µCi of labeled l-methionine and l-cystine was added, and the cells were incubated at 37 °C for 20 min (pulse). Radioactive medium was removed, and cells were quickly washed once with 2 ml of DMEM supplemented with 10% FBS, penicillin/streptomycin, 2 mM l-methionine, and 2 mM l-cysteine and then incubated in 4 ml of the same medium at 37 °C for the indicated chase times. Following the chase, cells were washed with 5 ml of ice-cold PBS and harvested in 0.5 ml of PBS. All traces of cells were removed with PBS washes before 100 µl of hot TSD buffer (50 mM Tris, pH 7.5, 1% SDS, 5 mM dithiothreitol) was added to solubilize ECM components. After scraping the plate, 1.2 ml of TNN buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) was then added to the plate, and the entire ECM extract was transferred to a tube for immunoprecipitation. One hundred µl of TSD was added to the cell pellets, and the mixture was boiled for 10 min. After centrifugation at 16,000 × g for 5 min at room temperature, this extract was added to 1.2 ml of TNN buffer for subsequent immunoprecipitation.

For immunoprecipitation, extracts were added to 30 µl of anti-HA-agarose (Sigma), incubated at 4 °C for 2 h, and washed five times with 1 ml of TNN buffer. Beads were resuspended and boiled in 2× SDS-PAGE sample buffer and proteins separated by SDS-PAGE. Gels were treated with Fluoro-Hance (Research Products International), dried, and exposed to x-ray film.

Immunofluorescence—HEK293T and AtT20 cells were cultured for 24 h following transfection on 18-mm coverslips pre-coated with 1 mg/ml polylysine (Sigma). Cells were washed with DMEM followed by PBS, fixed in 4% paraformaldehyde for 15 min, and then permeabilized for 15 min in 0.1% Triton X-100 in PBS. After 1 h of blocking in 5% bovine serum albumin, cells were immunostained for 1 h with mouse monoclonal anti-HA (clone HA-7, Sigma, 1:2000 dilution), rabbit polyclonal anti-CPE C terminus (1:2500 dilution), and rabbit polyclonal anti-calreticulin (Abcam, 1:1000 dilution). The cells were washed three times with 0.2% Tween 20 in PBS and then incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, 1:100 dilution) for 1 h. After five washes with 0.2% Tween 20 in PBS, coverslips were mounted in a small amount of Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Molecular Probes).

Carboxypeptidase Assays—For all assays, HEK293T cells were plated in 6-well plates and transfected after 24 h with either CPA6 expression plasmid or with pcDNA3.1 alone.
were removed 24–48 h after transfection, and the wells were washed three times with 50 mM Tris, pH 7.5, 150 mM NaCl. Visual inspection showed the absence of cells after this wash procedure. Substrate (1 ml per well) was added, and the plates were incubated with shaking at 37 °C in a humid chamber for the indicated time. All assays compared the ECM from plates transfected with CPA6 expression plasmid with plates transfected with vector alone.

The 3-(2-furyl)acryloyl-peptide (fa) substrates (Bachem) were typically dissolved in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl to a concentration of 0.5 mM. Cleavage of the substrate was measured by a decrease in absorbance at 336 nm. In determining the pH optimum, substrate was dissolved in 50 mM Tris acetate buffer containing 150 mM NaCl at the indicated pH values. Potato carboxypeptidase inhibitor was dissolved in water and benzylsuccinic acid was dissolved in Me2SO. These inhibitors were diluted to the indicated final concentration in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.5 mM fa-FF substrate, and incubated with CPA6 for 1 h at 37 °C. All experiments involving fa substrates were replicated in 2–3 separate experiments, most performed in duplicate or triplicate.

Synthetic peptides were either commercially obtained (Bachem) or custom-synthesized. They were dissolved in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl at a concentration of 0.5 mM, and 200-μl aliquots were incubated with ECM from CPA6 or control cells for lengths of time ranging from 100 to 1000 min. Cleavage of peptides was monitored using matrix-assisted laser desorption time-of-flight-mass spectrometry (MALDI-TOF-MS). Synthetic peptide solutions were desalted using a C18 ZipTip (Millipore) following the manufacturer’s protocol. The peptides were eluted with 1/70% acetonitrile, 0.1% trifluoroacetic acid, mixed with an equal volume of a saturated solution of α-hydroxycinnamic acid in the same solvent, and placed on a MALDI plate. Mass spectrometric analysis was carried out using a Voyager-DE STR (Applied Biosystems) in the positive ion mode: 2 kV accelerating voltage; 0.015% guide wire voltage; and laser intensity of 1847. All analysis of CPA6 cleavage of peptides was replicated in 2–3 separate experiments.

RESULTS

CPA6 is predicted to be a secreted protein, based on the presence of a signal peptide and the absence of any transmembrane domains that would cause the retention of the protein within the cell or on the cell surface. Other metallo-carboxypeptidases such as CPZ are also predicted to be soluble but are retained by the ECM. To investigate whether CPA6 is secreted from cells, HEK293T cells were transiently transfected with human CPA6 cDNA, and 2 days later the cells were fractionated and examined by Western blot. To detect CPA6, either an HA tag, a His4 tag, or both an HA and a His4 tag were included on the C terminus; comparable results were obtained in three independent experiments.

FIGURE 1. CPA6 is secreted as part of the extracellular matrix. HEK293T cells were transfected with empty vector (−) or plasmid expressing CPA6 (+). A, media, extracellular matrix, and cell fractions were collected and equal amounts separated by SDS-PAGE and immunoblotted with an antibody that recognizes the HA epitope. B, cells were transfected as above and treated with or without heparin, followed by SDS-PAGE and immunoblotting of indicated fractions with antibodies that recognize the HA or His4 epitopes. C, model of CPA6 is shown, indicating the polarized nature of CPA6 with a large preponderance of basic residues, through which CPA6 may interact with the acidic ECM, on the side opposite that of the active site. The model was generated with the online automated protein modeling program, Swiss-Model, using the crystal structure of human procarboxypeptidase B (entry 1KWM of the Protein Data Bank) as the template. Basic residues are indicated in blue, acidic residues in red, polar residues in yellow, and nonpolar residues in white. For the data shown in A and B, comparable results were obtained in three independent experiments.
CPA6 was also present (Fig. 1A, 6th lane). In the detergent/high salt extract, an additional band of ~37 kDa was detected; this corresponds to the predicted size of the mature form of CPA6 after propeptide removal. In the low salt extract, a cross-reacting protein of 37 kDa was present in the mock-transfected cells, so it was not possible to tell if a small additional amount of the mature form of CPA6 was also present in this fraction. Neither the 50-kDa nor the 37-kDa forms of CPA6 were detected in the medium (Fig. 1A, 2nd lane). Instead, a large amount of 37-kDa CPA6 protein was detected in the ECM fraction (Fig. 1A, 8th lane). The addition of 400 µg/ml heparin to the cells blocked the attachment of CPA6 to the ECM and led to the appearance of the protein in the medium (Fig. 1B, compare 3rd and 6th lanes). As heparin and related glycoproteins are negatively charged, they might interact with positively charged proteins such as CPA6, which has a predicted pI of 9.9. Modeling of human CPA6 shows that the majority of basic residues are located on the face of the molecule opposite that of the active site (Fig. 1C), suggesting that this may orient CPA6 with respect to the ECM.

The upstream protease responsible for processing of CPA6 into the 37-kDa active form was previously proposed to be furin or a related proprotein convertase (20). To investigate this possibility, HEK293T cells were co-transfected with plasmids expressing CPA6 as well as α1-PDX, an inhibitor previously characterized to be specific for furin and related proprotein convertases (21). The co-transfection of inhibitor resulted in a reduction of the 37-kDa CPA6 secreted into the ECM, whereas the secretion of the 50-kDa pro-CPA6 was dramatically increased (Fig. 2A). Transfection of 2–8 times more plasmid expressing α1-PDX did not result in an increase in secreted 50-kDa pro-CPA6 (Fig. 2A). To investigate whether the processing of CPA6 occurred inside or outside of the cell, transfections of CPA6 and α1-PDX were performed in separate cells, and the cells were mixed 6 h following transfection. Cells were then co-cultured for an additional 24 h prior to Western blot analysis of the CPA6 forms. No change in the processing of CPA6 was observed when cells expressing α1-PDX were mixed with cells expressing CPA6, suggesting that propeptide removal from CPA6 occurs within the cells (Fig. 2B).

The time course of the appearance of CPA6 in the ECM was examined by pulse-chase analysis with [35S]-Met/Cys. Cell- and ECM-labeled extracts collected at different time points were immunoprecipitated with an anti-HA antibody to pull down HA-tagged CPA6. Pro-CPA6 was detected in the cell extracts at all time points, generally as a doublet that likely reflects glycosylation of CPA6 (Fig. 3A). No processed 37-kDa CPA6 was detected in the cells in most experiments, although a very weak band of this size was detected in one experiment at the 40- and 100-min time points (data not shown). In the ECM extracts 50-kDa pro-CPA6 was detected at the 20–120-min time points with decreasing intensity, whereas 37-kDa CPA6 was detected at 60, 120, and overnight time points with a peak at 120 min. To further test whether the 50-kDa form was converted into the 37-kDa form in the ECM without the contribution of cells, the pulse-chase experiment was repeated as above, except that the cells were removed from some of the plates after 20 min of chase. In the absence of cells, the 50-kDa form was not converted to the 37-kDa form after 100 min (data not shown). This confirms the results of α1-PDX treatment indicating that the formation of the 37-kDa form occurs within the cell.

The time required for secretion of CPA6 from the HEK293T cells is somewhat slower than typical constitutive secretion. This slow rate of exit could be due to the retention of the protein in the ER and/or Golgi. To address this, immunofluorescence was performed on transfected HEK293T cells. The majority of the CPA6 showed a broad diffuse distribution reminiscent of the ER (Fig. 3B). Co-staining with calreticulin, an ER marker, confirmed the ER distribution of CPA6 (Fig. 3B).

Because CPA6 mRNA was previously found to be present in neuronal tissue (20), and neurons contain both regulated and constitutive secretory pathways, it was important to examine the routing of CPA6 in a cell line that contains both of these pathways. Previously, AtT20 cells have been used to examine sorting into the regulated secretory pathway (22, 23). When expressed in the AtT20 cell line, CPA6 shows a perinuclear distribution (Fig. 3C). The distribution of CPA6 in the AtT20 cells is less diffuse than found in HEK293T cells and does not co-localize with calreticulin (Fig. 3C). Although a small amount of CPA6 is found in the tips of the AtT20 cells, this does not appear to co-localize with CPE, a marker of the regulated secre-
CPA6 Functions in the ECM

A.

B.

C.

FIGURE 3. Processed CPA6 is not retained within the cell. A, HEK293T cells were transfected with empty vector (−) or plasmid expressing HA- and His6-tagged CPA6 (+). Pulse-chase analysis was performed using 35S-labeled Met/Cys. The label was chased with unlabeled media for the indicated times in minutes, or overnight (ON), followed by extraction of cellular and ECM proteins. Tagged CPA6 was immunoprecipitated from extracts and resolved by SDS-PAGE, and the gels were dried and exposed to film for 20 days. Similar results were obtained in three independent experiments. HEK293T (B) or AtT20 (C) cells were transfected with a plasmid expressing HA-and His6-tagged CPA6. Immunocytochemistry was performed with antibodies directed against the HA epitope and calreticulin (B), and an epitope within the C terminus of CPE (C). Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI).

dictory pathway (Fig. 3C). Thus, CPA6 does not appear to traffic to the cell surface via the regulated pathway.

Previously, modeling suggested that CPA6 cleaves peptides with hydrophobic C-terminal amino acids. To investigate whether CPA6 in the ECM was enzymatically active, we transfected HEK293T cells with CPA6, removed the cells after 1–2 days of incubation, and incubated the ECM with a series of fa substrates, which undergo a decrease in absorbance at 336 nm days of incubation, and incubated the ECM with a series of fa substrates. Tagged CPA6 was immunoprecipitated from extracts and resolved by SDS-PAGE, and the gels were dried and exposed to film for 20 days. Similar results were obtained in three independent experiments. HEK293T (B) or AtT20 (C) cells were transfected with a plasmid expressing HA-and His6-tagged CPA6. Immunocytochemistry was performed with antibodies directed against the HA epitope and calreticulin (B), and an epitope within the C terminus of CPE (C). Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI).

tory pathway (Fig. 3C). Thus, CPA6 does not appear to traffic to the cell surface via the regulated pathway.

Accordingly, when the samples of media and ECM from cells transfected with HA- and His6-tagged CPA6 were probed with antibodies to either the HA tag or the His6 tag, the relative levels of immunoreactive CPA6 in the fractions were not identical; less His6 immunoreactivity was detected in the ECM fraction than HA immunoreactivity relative to the amounts of these signals in the media fraction (Fig. 1B). Because the His6 tag was located on the C terminus of the construct, following the HA tag, one possible explanation of this observation was that CPA6 cleaved the His residues from the C terminus. To test if the His6 tail of CPA6 undergoes autocleavage (either intramolecular or intermolecular), a point mutation was created that rendered the enzyme inactive by replacing the active site Glu270 (CPA1 numbering) with a Gln. ECM from cells expressing either this inactive mutant or the active enzyme were compared by Western blotting. A strong His6 immunoreactive band was detected for concentration used for these studies approximated the enzyme $K_m$ for fa-FF, and so the $K_i$ for these inhibitors will be approximately half the observed $IC_{50}$. Therefore, the $K_i$ for benzylsuccinic acid inhibition of CPA6 is 3–4 μM, which is about 10-fold higher than the 0.28 μM $K_i$ determined for CPA1 (24). Similarly, the $K_i$ for potato carboxypeptidase inhibitor inhibition of CPA6 is 20–30 nM, compared with a 1.5 nM $K_i$ for CPA1 (25).

The above results showed that CPA6 is secreted into the ECM where it cleaves C-terminal hydrophobic amino acids from small synthetic substrates (Fig. 1A and Fig. 4A). Interestingly, when the samples of media and ECM from cells transfected with HA- and His6-tagged CPA6 were probed with antibodies to either the HA tag or the His6 tag, the relative levels of immunoreactive CPA6 in the fractions were not identical; less His6 immunoreactivity was detected in the ECM fraction than HA immunoreactivity relative to the amounts of these signals in the media fraction (Fig. 1B). Because the His6 tag was located on the C terminus of the construct, following the HA tag, one possible explanation of this observation was that CPA6 cleaved the His residues from the C terminus. To test if the His6 tail of CPA6 undergoes autocleavage (either intramolecular or intermolecular), a point mutation was created that rendered the enzyme inactive by replacing the active site Glu270 (CPA1 numbering) with a Gln. ECM from cells expressing either this inactive mutant or the active enzyme were compared by Western blotting. A strong His6 immunoreactive band was detected for...
MARCH 14, 2008 • VOLUME 283 • NUMBER 11

**CPA6 Functions in the ECM**

**CPA6 is inhibited by selective carboxypeptidase inhibitors.** HEK293T cells, either untransfected or transfected with plasmid expressing HA- and His6-tagged CPA6, were removed 2 days following transfection, and the ECM remaining on the plate was incubated for 1 h with 0.5 mM fa-FF at 37 °C and pH 7.5. Benzylsuccinic acid (BZS) (A) and potato carboxypeptidase inhibitor (PCI) (B) were added to the substrate at the indicated concentrations. The change in substrate absorbance at 336 nm was measured, and the difference between CPA6-expressing and control cell ECM was determined and plotted as a percentage of the change observed with uninhibited enzyme. The experiment was performed twice in triplicate. One experiment is shown with error bars indicating standard deviation. Points without error bars had standard deviations smaller than the symbol size.

**CPA6 is able to cleave amino acids from the C termini of proteins.** A, schematic of the structure of CPA6 and its tags used in this experiment, showing the N-terminal signal peptide (SP), the propeptide (Pro), the carboxypeptidase domain, and the HA and His6 (H6) tags followed by one or more additional amino acids (X). B, HEK293T cells were transfected with a plasmid expressing the above CPA6 protein with the terminating amino acid indicated on the bottom panel by its one-letter code (SH indicates five additional histidines and HF indicates an additional dipeptide following the six histidines). Two constructs did not contain an additional amino acid on the C terminus after the His6 tag; one was a point mutation that converted the critical active site residue Glu398 to Gln (−−− on bottom of figure), the other was wild type active CPA6 (+ + + on bottom of figure). ECM proteins were solubilized and resolved by SDS-PAGE followed by Western blotting with anti-HA and anti-His6 antibodies. Band quantification was performed with ImageJ software. The ratio of anti-His6 band intensity (His tag remaining) to anti-HA band intensity (total protein) was determined and converted to a percentage of the ratio obtained from inactive enzyme (100% of His tag remaining).

In two cases more than one amino acid was added, resulting in a dipeptide His-Phe (final sequence HHHHHHF) and a pentapeptide containing five additional histidines, for a total of 11 His. These various constructs were transfected into HEK293T cells, and the ECM was analyzed by Western blotting with both HA and His6-specific antisera (Fig. 6B). His6 immunoreactivity was then normalized against HA immunoreactivity, relative to that detected for inactive CPA6 (Fig. 6C). The His6 tag was cleaved from CPA6 with C-terminal Leu, Met, Trp, and Phe as efficiently as CPA6 terminating in the His6 tag alone. Also, the construct containing 7 His followed by a Phe (HF in Fig. 6C) was cleaved as efficiently as the construct containing 6 His followed by a Phe (F in Fig. 6C). Several other constructs showed intermediate cleavage of the His6 tag (C-terminal Ile, Asn, and Val, Gln, Ala, and the construct containing 11 His). The remainder of the constructs tested (C-terminal Ser, Tyr, Thr, Glu, and Asp) exhibited little or no cleavage of the His6 tag.

A series of peptides was analyzed for cleavage by CPA6. Many of these peptides are biologically active and present in the olfactory bulb where CPA6 is located, whereas other peptides tested represent the N or C termini of proteins and are not likely to be physiological substrates for CPA6. These latter peptides were used simply to gain further knowledge about the cleavage specificity of CPA6. Peptides were incubated for a range of time (from 100 to 1000 min) at 37 °C with ECM secreted from CPA6-expressing cells or from control cells. Cleavage was detected by analysis using MALDI-TOF mass spectrometry. A sample of results obtained is shown in Fig. 7, and Table 1 shows a complete list of peptides and cleavages detected. Greater than 70% of Met-enkephalin-Arg-Phe, Big SAAS, and aldolase C terminus peptide were found to be C-terminally processed by CPA6...
after a 1000-min incubation. Neurotensin, Little SAAS, and CPE C terminus peptides were only partially processed in the same incubation period. Met- and Leu-enkephalin, fly CPD tail2 C terminus, and angiotensin I (as well as the related [des-Asp^1]angiotensin I) were weakly processed by CPA6. It appears that the penultimate His of angiotensin I is readily cleaved upon removal of the C-terminal Leu, as only minor amounts of the intermediate were detected (Fig. 7), even upon short incubations of either angiotensin I or [des-Asp^1]angiotensin I with CPA6 (data not shown).

**DISCUSSION**

It was previously hypothesized that CPA6 was a secreted protein based on the presence of a signal peptide and the lack of a transmembrane domain. In this study we have confirmed that CPA6 is secreted. The finding that CPA6 does not co-localize...
with CPE suggests that CPA6 is routed into the constitutive secretory pathway and not the CPE-containing regulated pathway in which peptide processing takes place. This, together with the localization of the active enzyme to the ECM and a neutral pH optimum (with little activity at pH values below 6), indicates that CPA6 is not an intracellular peptide-processing enzyme. The neutral pH optimum of CPA6 is more consistent with a function outside of the cell in the ECM. Pulse-chase analysis confirmed that processed CPA6 is not retained inside the cell for significant periods of time but is secreted upon processing to its active 37-kDa form. The polarized nature of the model for CPA6 suggests that CPA6 may orient itself with respect to the negatively charged glycoproteins of the ECM so that its active site remains accessible for potential substrates. Interestingly, this charge polarity has also been found for rat mast cell carboxypeptidase, and this polarity has been proposed to be involved in the orientation of the interaction of the protein with the heparin matrix of the mast cell granules (26).

Pro-CPA6 was predicted to be processed into active enzyme by the action of furin or a furin-like endoprotease (proprotein convertase 5, proprotein convertase 7, or PACE4) that cleaves at the RNRR sequence separating the pro-domain and the active CP domain of human, rat, and mouse CPA6 (13, 20). One or more of these endopeptidases is present in the secretory pathway of most mammalian cells, where they are thought to function in the trans-Golgi network as well as on the cell surface (21, 27, 28). Co-transfection of HEK293T cells with α1-PDX, an inhibitor specific for furin and related proprotein convertases, confirmed that pro-CPA6 is processed to some extent by one or more of these proteases, presumably within the cell. We have also shown through an in vitro digest that purified furin is able to convert the 50-kDa form of CPA6 into the 37-kDa form. Together, these data suggest that furin and/or a related proprotein convertase cleave pro-CPA6 into CPA6 within the cell.

It was previously hypothesized that CPA6 cleaves C-terminal hydrophobic residues, based on amino acid sequence similarity to other CPAs and modeling of the three-dimensional structure of CPA6 (13). In this study, synthetic carboxypeptidase substrates, peptides, and protein substrates were used to analyze the substrate specificity of CPA6. The synthetic substrates of the 3-(2-furyl)acylloxy-leupeptin series enabled us to determine that CPA6 preferentially cleaves large hydrophobic amino acids as predicted, with no cleavage of basic amino acids Lys and Arg. This was confirmed through the use of peptide substrates that extended this lack of cleavage to acidic amino acids. The penultimate residue of the substrate also imparts specificity, as Pro and Gly are unfavorable in this position. It is interesting to note that, in the case of neurotensin, CPA6 does not cleave the penultimate Ile after removal of the C-terminal Leu. The reason for the absence of this second cleavage is not clear, but it could be due to due secondary structure.

CPA6 was able to cleave the C-terminal His6 tag. This cleavage of His has also been observed for CPA1 and mast cell CPA (CPA3). In the case of CPA1 His cleavage has been exploited as a method to remove His6 tags from the C terminus of recombinant proteins (29). CPA3 has been shown to be able to cleave His10 of [des-Leu10]neurotensin I in a similar manner as that observed in this study for CPA6 (30). The cleavage of the C-terminal His tag is not an intramolecular reaction, based on the finding that co-transfection of catalytically inactive CPA6 together with the active enzyme resulted in cleavage of the His6 tag from the inactive enzyme. Thus, CPA6 is able to remove amino acids from protein substrates as well as synthetic and peptide substrates. This cleavage of the C-terminal His6 tag was exploited as a way of determining substrate specificity through the addition of an additional “blocking” residue. The results of these analyses largely agreed with and extended the results from cleavage of synthetic substrates and peptides. In addition to Phe and Leu, CPA6 was found to be able to efficiently cleave C-terminal His, Trp, and Met. Interestingly, Tyr would be expected to be cleaved but is not in this assay, whereas Val and Ile were previously predicted to be good substrates for CPA6 (13) but are weak substrates in this assay. Assays that used small synthetic substrates and peptides found that larger hydrophobic amino acids (including Tyr) are substrates for CPA6. Weak cleavage of Tyr from the C terminus of the protein may be due to interfering secondary structure.

The biological function of CPA6 is of interest. Previously, based on the finding that a patient with Duane syndrome has a mutation within the CPA6 gene and on the localization of CPA6 expression in the embryonic mouse eye muscle, it was hypothesized that CPA6 processes an axonal guidance factor. Although in adult mouse brain CPA6 mRNA is abundant only in the olfactory bulb mitral and granular cell layers, it is more broadly expressed in embryonic brain and other tissues. The present observations that CPA6 is enriched in the ECM and is able to cleave the C termini of a number of biologically active peptides raise the possibility that CPA6 functions in the extracellular processing of neuropeptides. One peptide processed by CPA6 is neurotensin; this peptide plays a role in locomotor activity, thermal regulation, stress, pain, drug addiction, and depression (31). The Allen Brain Atlas indicates that neurotensin mRNA is expressed in the mitral cell layer of the olfactory bulb, whereas mRNA for the NTR1 neurotensin receptor is

### Table 1

| Peptide | Sequence and site of cleavage |
|---------|------------------------------|
| >70% cleavage |                      |
| Met-enkephalin-Arg-Phe | YGGFPR ↓ F |
| Big SAAS | ARPKVEPSRLASAAPLAETSTPLR ↓ L |
| Mouse aldolase C terminus | LYTANHA ↓ Y |
| 20–70% cleavage |                         |
| Neurotensin | pELYNKPRPPYI ↓ L |
| Little SAAS | SSAAPLAETSTPLR ↓ L |
| CPA C terminus | EMGSEFLN ↓ F |
| <20% cleavage |                  |
| Leu-enkephalin | YGF ↓ L |
| Met-enkephalin | YGP ↓ M |
| Angiotensin I | DRYYIHFP ↓ H ↓ L |
| CPA5 C terminus | RYYYHFP ↓ H ↓ L |
| Fly CPD tail 2 C terminus | CVMLGNKSNK ↓ H |
| No cleavage |                  |
| PEN | SVGDQLGPEVPPENVLGALLRV |
| Big LEN | LENSSPOAPARLLPP |
| Bradykinin | RPPGFSPP |
| [Des-Arg9]bradykinin | RPPGFSPP |
| CPA5 C terminus | QTIMKHNTH HPV |
| YE-17 | PYPENVNLGALLVRKLE |
| CPA-mid | YDPDRPFCRRNEDD |

*Peptides were incubated at a 0.5 mM concentration with ECM from WT cells and with ECM from cells expressing CPA6 for 1000 min at 37 °C and pH 7.5.*
found predominantly in the inner granular layer with weak expression found in most parts of the olfactory bulb. A report by Moyse et al. (32) in which 125I-neurotensin was used to identify neurotensin binding in sections of rat brain indicated neurotensin binding in the external plexiform and granular layers of the olfactory bulb. A review by Kitabgi (33) has summarized the evidence for inactivation of neurotensin by endopeptidases 24.11, 24.15, and 24.16 to produce neurotensin 1–8, neurotensin 1–10, and neurotensin 1–11. Interestingly, cleavage of neurotensin by CPA6 produces neurotensin 1–12. This cleavage inactivates neurotensin as integrity of the C-terminal hexapeptide 8–13 of neurotensin is necessary for strong receptor binding (34). A neurotensin 1–12-like immunoreactivity was detected in experiments using a high pressure liquid chromatography-radioimmunoassay procedure to detect neurotensin in rat brain extracts, although the origin of this neurotensin fragment was not discussed (35). CPA6 may be responsible for inactivating neurotensin to neurotensin 1–12 within the olfactory bulb.

Other peptides found to be cleaved by CPA6 in this study include Met- and Leu-enkephalin. Any cleavage of enkephalin greatly reduces the affinity of the peptide for the opioid receptors. The extracellular processing of enkephalin is primarily thought to involve two enzymes as follows: aminopeptidase N that removes the N-terminal Tyr and neutral endopeptidase (endopeptidase 24.11) that cleaves between the Gly-Phe bond to produce Tyr-Gly-Gly and the C-terminal dipeptide (36–38). There is some evidence that Leu-enkephalin is cleaved by a CP within the brain; a small amount of Leu was released upon incubation of Leu-enkephalin with brain membranes (39). However, previous studies did not specifically examine olfactory bulb (37, 38), and it is possible that CPA6 plays a major role in enkephalin degradation in this brain region. Interestingly, proenkephalin mRNA is present at high levels in the mouse olfactory bulb (Allen Brain Atlas), and enkephalin peptides and opioid receptors have also been detected in this brain region (40, 41). The function of olfactory bulb enkephalin is not clear; in other regions enkephalin has a role in analgesia and the reward pathways (36). Additional roles for enkephalin include synaptogenesis and neurite outgrowth (42, 43), and it is possible that olfactory bulb enkephalin is involved in this function.

Unlike the inactivation of neurotensin and enkephalin by CPA6, the cleavage of angiotensin I by CPA6 would result in the activation of this peptide. Angiotensin I is inactive and can be converted to active angiotensin II by several mechanisms, the most commonly known being angiotensin-converting enzyme-1 (ACE1) (44). Angiotensinogen mRNA is present at high levels in some subregions of the olfactory bulb, and mRNAs for angiotensin receptor 1a and angiotensin receptor 2 are present at moderate levels, whereas ACE1 mRNA is very low in the olfactory bulb (Allen Brain Atlas). Immunoreactive angiotensinogen was also detected in olfactory bulb (45). We have shown here that angiotensin I can be cleaved by CPA6 to produce angiotensin II. It appears that the limiting factor in this cleavage is the removal of Leu10, as extremely small amounts of angiotensin 1–9 are detected relative to the amount of angiotensin II (i.e. angiotensin 1–8). Thus, removal of His9 appears to proceed faster than removal of Leu10. A possibility in the production of angiotensin II exists in which angiotensin-convert- ing enzyme-2 (ACE2) removes Leu10 of angiotensin I followed by the removal of His9 by CPA6. ACE2 is widely distributed throughout the brain, including the olfactory bulb, but is incapable of removing His9 to produce angiotensin II (46). The function of angiotensin within the brain is in the regulation of water and sodium intake and the control of sympathoadrenal systems (47). However, it may also have a role in brain development, neuron migration, and the processing of sensory information through the brain (47).

In summary, our findings establish that CPA6 is present in the ECM and able to cleave a broad range of peptide and protein substrates. Taken together with previous studies showing the distribution of CPA6 in the brain and in embryonic tissue, and the finding that disruption of the CPA6 gene is linked to Duane syndrome, it is likely that CPA6 plays an important role in the activation and/or inactivation of signaling molecules.

Acknowledgments—Microscopy was performed in the laboratory of Dr. Jonathan Backer, Department of Molecular Pharmacology, Albert Einstein College of Medicine. We thank Dr. Gary Thomas (Vollum Institute, Portland, OR) for providing the plasmid expressing the proprotein convertase inhibitor α1-PDX.

REFERENCES

1. Arolas, J. L., Vendrell, J., Aviles, F. X., and Fricker, L. D. (2007) Curr. Pharm. Des. 13, 347–364
2. Thomas, A. G., Wozniak, K. M., Tsukamoto, T., Calvin, D., Wu, Y., Rojas, C., Vornov, J., and Slusher, B. S. (2006) Adv. Exp. Med. Biol. 576, 327–337
3. Rodriguez de la Vega, M., Sevilla, R. G., Hermoso, A., Lorenzo, J., Tanco, S., Diez, A., Fricker, L. D., Bautista, J. M., and Aviles, F. X. (2007) FASEB J. 21, 851–865
4. Aviles, F. X., Vendrell, J., Guasch, A., Coll, M., and Huber, R. (1993) Eur. J. Biochem. 211, 381–389
5. Fricker, L. D. (1988) Annu. Rev. Physiol. 50, 309–321
6. Fricker, L. D. (2004) in Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) 2nd Ed., Vol. 1, pp. 840–844, Academic Press, San Diego
7. Fricker, L. D. (2004) in Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) 2nd Ed., Vol. 1, pp. 848–851, Academic Press, San Diego
8. Fricker, L. D. (2007) Endocrinology 148, 4185–4190
9. Kalinina, E., Biswas, R., Berezniuk, I., Hermoso, A., Aviles, F. X., and Fricker, L. D. (2007) FASEB J. 21, 836–850
10. Fernandez-Gonzalez, A., La Spada, A. R., Treadaway, J., Higdon, J. C., Harris, B. S., Sidman, R. L., Morgan, J. I., and Zuo, J. (2002) Science 295, 1904–1906
11. Mulliken, R. J., Eicher, E. M., and Sidman, R. L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 208–212
12. Bouma, B. N., and Mosnier, L. O. (2006) Annu. Med. 38, 378–388
13. Wei, S., Segura, S., Vendrell, J., Aviles, F. X., Lanoue, E., Day, R., Feng, Y., and Fricker, L. D. (2002) J. Biol. Chem. 277, 14954–14964
14. Huang, H., Reed, C. P., Zhang, J. S., Shridhar, V., Wang, L., and Smith, D. I. (1999) Cancer Res. 59, 2981–2988
15. Pizzuti, A., Calabrese, G., Bozzali, M., Telvi, L., Morizio, E., Guida, V., Gatta, V., Stuppa, L., Ion, A., Palka, G., and Dallapiccola, B. (2002) Investig. Ophthalmol. Vis. Sci. 43, 3609–3612
16. Calabrese, G., Telvi, L., Capodiferro, F., Morizio, E., Pizzuti, A., Stuppa, L., Bordoni, R., Ion, A., Fantasia, D., Mingarelli, R., and Palka, G. (2000) Eur. J. Hum. Genet. 8, 319–324
17. Calabrese, G., Stuppa, L., Morizio, E., Guanciali Franchi, P., Pompetti, F., Mingarelli, R., Marsilio, T., Rocchi, M., Gallenga, P. E., Palka, G., and Dallapiccola, B. (1998) Eur. J. Hum. Genet. 6, 187–193
18. Vincent, C., Kalatzis, V., Compain, S., Levilliers, I., Slim, R., Graia, F., Pereira, M. L., Nivelon, A., Croquette, M. F., Lacombe, D., Vigneron, I., Helias, J., Broyer, M., Callen, D. F., Haan, E. A., Weissenbach, J., Lacroix, B., Bellane-Chantelot, C., Le Paslier, D., Cohen, D., and Petit, C. (1994) *Hum. Mol. Genet.* **3**, 1859–1866

19. Gutowski, N. J. (2000) *Eur. J. Neurol.* **7**, 145–149

20. Fontenele-Neto, J. D., Kalinina, E., Feng, Y., and Fricker, L. D. (2005) *Brain Res. Mol. Brain Res.* **137**, 132–142

21. Thomas, G. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 753–766

22. Varlamov, O., and Fricker, L. D. (1996) *J. Biol. Chem.* **271**, 6077–6083

23. Ciccotosto, G. D., Schiller, M. R., Eipper, B. A., and Mains, R. E. (1999) *J. Cell Biol.* **144**, 459–471

24. Lee, M., Jin, Y., and Kim, D. H. (1999) *Bioorg. Med. Chem.* **7**, 1755–1760

25. Molina, M. A., Marino, C., Oliva, B., Aviles, F. X., and Querol, E. (1994) *J. Biol. Chem.* **269**, 21467–21472

26. Cole, K. R., Kumar, S., Trong, H. L., Woodbury, R. G., Walsh, K. A., and Neurath, H. (1991) *Biochemistry* **30**, 648–655

27. Seidah, N. G., and Prat, A. (2002) *Essays Biochem.* **38**, 79–94

28. Steiner, D. F. (1998) *Curr. Opin. Chem. Biol.* **2**, 31–39

29. 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

30. Lundequist, A., Tchougounova, E., Abrink, M., and Pejler, G. (2004) *J. Biol. Chem.* **279**, 32339–32344

31. Geisler, S., Berod, A., Zahm, D. S., and Rostene, W. (2006) *Peptides (N. Y.)* **27**, 2364–2384

32. Moyse, E., Rostene, W., Vial, M., Leonard, K., Mazella, J., Kitabgi, P., Vincent, J. P., and Beaudet, A. (1987) *Neuroscience* **22**, 525–536

33. Kitabgi, P. (2006) *Peptides (N. Y.)* **27**, 2515–2522

34. Mazella, J., Chabry, J., Kitabgi, P., and Vincent, J. P. (1988) *J. Biol. Chem.* **263**, 144–149

35. Bennett, G. W., Moss, S. H., Forster, C. D., and Marsden, C. A. (1998) *Brain Res. Dev. Brain Res.* **111**, 189–196

36. Goodman, R. R., Fricker, L. D., and Snyder, S. H. (1983) in *Brain Peptides* (Krieger, D. T., Brownstein, M. J., and Martin, J. B., eds) pp. 827–849, John Wiley & Sons, Inc., New York

37. Vogel, Z., and Altstein, M. (1977) *FEBS Lett.* **80**, 332–336

38. Meek, J. L., Yang, H. Y., and Costa, E. (1977) *Neuropharmacology* **16**, 151–154

39. Ollanas, M. C., and Onali, P. (1992) *Mol. Pharmacol.* **42**, 109–115

40. Bogan, N., Brecha, N., Gall, C., and Karten, H. J. (1982) *Neuroscience* **7**, 895–906

41. Davila-Garcia, M. I., and Azmitia, E. C. (1990) *Adv. Exp. Med. Biol.* **265**, 75–92

42. Escobar, M. L., Barea-Rodriguez, E. I., Derrick, B. E., Reyes, J. A., and Martinez, J. L., Jr. (1997) *Brain Res.* **751**, 330–335

43. Karamyan, V. T., and Speth, R. C. (2007) *Regul. Pept.* **143**, 15–27

44. Genain, C. P., Van Loon, G. R., and Kotchen, T. A. (1985) *J. Clin. Investig.* **76**, 1939–1945

45. Doobay, M. F., Talman, L. S., Obr, T. D., Tian, X., Davison, R. L., and Lazartigues, E. (2007) *Am. J. Physiol.* **292**, R373–R381

46. Saavedra, J. M. (2005) *Cell. Mol. Neurobiol.* **25**, 485–512