Carboxypeptidase O Is a Glycosylphosphatidylinositol-anchored Intestinal Peptidase with Acidic Amino Acid Specificity*[S]

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Background: All previously characterized metallocarboxypeptidases of the A/B subfamily are secreted enzymes that cleave aliphatic or basic residues and are initially produced as inactive proenzymes.
Results: Carboxypeptidase O is a membrane-anchored intestinal enzyme that cleaves acidic residues and is not made from a proenzyme.
Conclusion: Carboxypeptidase O is distinct from other metallocarboxypeptidases.
Significance: Carboxypeptidase O plays a unique physiological role in the intestinal release of acidic amino acids from dietary peptides and proteins.

The first metallocarboxypeptidase (CP) was identified in pancreatic extracts more than 80 years ago and named carboxypeptidase A (CPA; now known as CPA1). Since that time, seven additional mammalian members of the CPA subfamily have been described, all of which are initially produced as proenzymes, are activated by endoproteases, and remove either C-terminal hydrophobic or basic amino acids from peptides. Here we describe the enzymatic and structural properties of carboxypeptidase O (CPO), a previously uncharacterized and unique member of the CPA subfamily. Whereas all other members of the CPA subfamily contain an N-terminal prodomain necessary for folding, bioinformatics and expression of both human and zebrafish CPO orthologs revealed that CPO does not require a prodomain. CPO was purified by affinity chromatography, and the purified enzyme was able to cleave proteins and synthetic peptides with greatest activity toward acidic C-terminal amino acids unlike other CPA-like enzymes. CPO displayed a neutral pH optimum and was inhibited by common metallocarboxypeptidase inhibitors as well as citrate. CPO was modified by attachment of a glycosylphosphatidylinositol membrane anchor to the C terminus of the protein. Immunocytochemistry of Madin-Darby canine kidney cells stably expressing CPO showed localization to vesicular membranes in subconfluent cells and to the plasma membrane in differentiated cells. CPO is highly expressed in intestinal epithelial cells in both zebrafish and human. These results suggest that CPO cleaves acidic amino acids from dietary proteins and peptides, thus complementing the actions of well known digestive carboxypeptidases CPA and CPB.

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The M14 family of metallocarboxypeptidases (CPs)[2] and CP-like proteins consists of 25 members in humans (1, 2). The major function of these enzymes is the removal of C-terminal amino acids from peptides and proteins during maturation and/or degradation (3, 4). One important area of carboxypeptidase function is in the digestion of dietary proteins and peptides to release amino acids that are able to be absorbed in the intestinal tract (5). This activity was identified more than 80 years ago when the first CP, originally called simply "carboxypeptidase," was found to be produced in large quantities by the pancreas and secreted into the intestine for dietary protein digestion (6). A second pancreatic carboxypeptidase was subsequently identified and named CPB for its substrate specificity toward basic C-terminal amino acids (7), and the first carboxypeptidase was renamed CPA for its aliphatic/aromatic amino acid specificity. This enzyme is now known as CPA1 because an additional pancreatic enzyme was discovered and named CPA2 (8). However, none of these enzymes cleave acidic C-terminal amino acids, raising the question of how peptides and proteins containing C-terminal aspartate and glutamate are digested in the intestine.

In addition to the three digestive carboxypeptidases identified in the exocrine pancreas, a number of carboxypeptidases have been identified in non-pancreatic tissues. Examples include CPN, which processes circulating bioactive peptides (9); CPE with a prominent role in neuropeptide maturation (10, 11); and many others (4, 12–17). The 25 members of the M14 carboxypeptidase family have been grouped into four subfamilies based on sequence and structural similarities (Fig. 1A). All subfamilies share a carboxypeptidase domain with critical active site and substrate-binding residues generally conserved between subfamilies (18). All characterized N/E members that are enzymatically active have substrate specificity toward basic

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.
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[2] The abbreviations used are: CP, metallocarboxypeptidase; MDCK, Madin-Darby canine kidney; CCP, cytosolic carboxypeptidase; GPI, glycosylphosphatidylinositol; hCPO, human CPO; zCPO, zebrafish CPO; dpf, day(s) post-fertilization; fa, 3-(2-furyl)acryloyl; PI-PLC, phosphatidylinositol-specific phospholipase C.
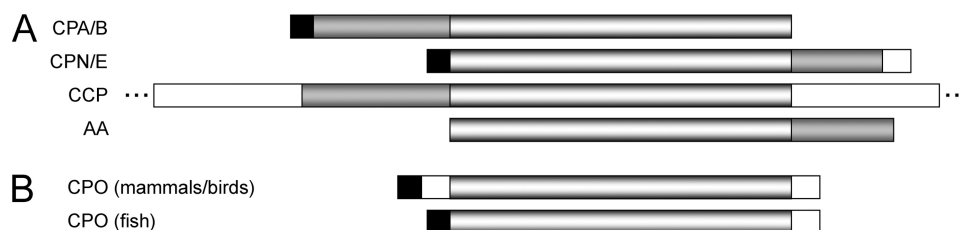
FIGURE 1. **Comparison of domain structure of CP subfamilies.** *A,* all M14 CPs have a 300-residue catalytic domain (*light gray*). Members of the A/B and cytosolic CCP subfamilies usually have an N-terminal β-sheet-rich domain (*dark gray*), whereas members of the N/E and aminoacylase (*AA*) subfamilies have a C-terminal β-sheet-rich domain (*dark gray*). Some CPs have signal peptides (*black*) and additional domains with uncharacterized structure (*white*). The CCPs vary in length (indicated by " . . ."). *B,* CPO consists of an N-terminal signal peptide, a catalytic domain, and a short C-terminal sequence. In mammals and birds, CPO also has a short peptide between the signal peptide and the CP domain; this sequence is not present in fish CPO.

C-terminal amino acids, and characterized A/B members exhibit aliphatic or basic amino acid specificity. Although no previously characterized mammalian A/B or N/E CPs have been shown to cleave acidic residues, a number of members of the cytosolic carboxypeptidase (CCP) (19) and aminoacylase (20) subfamilies are able to cleave substrates with C-terminal aspartate or glutamate residues. All members of the A/B and N/E subfamilies contain N-terminal signal peptides that direct the proteins into the endoplasmic reticulum and secretory pathway, whereas all members of the CCP and aminoacylase subfamilies lack signal peptides and are expressed in the cytosol. Two CPs are known to be membrane-bound; CPD contains a transmembrane domain (21), and CPM is membrane-bound through a glycosylphosphatidylinositol (GPI) anchor (22).

Several years ago, a new member of the A/B subfamily was identified through a bioinformatics analysis of the human genome and named carboxypeptidase O (CPO) (15). Modeling predicted CPO to have specificity for acidic C-terminal amino acids. However, the N terminus of the protein was not identified, and it was thought that CPO might be a pseudogene. To address this, we used a bioinformatics approach to identify full-length CPO from a number of species; all showed the presence of a signal peptide but either no prodomain (zebrafish) or a very short N-terminal extension (mammals; Fig. 1*B*). Because the dogma in the field was that the prodomain was essential for folding of proteins in this subfamily, CPO was predicted to be inactive. We show in this report that CPO is functional without the presence of a prodomain and is enzymatically active with acidic amino acid specificity. CPO is membrane-attached via a GPI anchor and is found on the apical surface of intestinal epithelial cells. We propose that CPO completes the complement of digestive enzymes within the intestine: CPA1 and CPA2 cleave aliphatic/aromatic amino acids, CPB1 cleaves basic amino acids, and CPO cleaves acidic amino acids.

EXPERIMENTAL PROCEDURES

Plasmids—The human CPO cDNA was purchased from Open Biosystems (clone ID 8327546; GenBank[TM] accession number BC112078). Human CPO was tagged on the C terminus with the HA or His$_6$ epitopes by PCR with the *Pfu* Ultra II polymerase (Stratagene) and subcloned into pcDNA3.1(+) for mammalian cell expression. Human CPO with the C-terminal His$_6$ tag (hCPO-His$_6$) was subcloned into the pVL1393 plasmid for baculovirus expression. The zebrafish CPO cDNA was amplified from cDNA made from 5-day postfertilization (dpf) zebrafish, tagged with the His$_6$ epitope as above, and subcloned

into the pCRII and pVL1393 plasmids. All cDNAs subcloned by PCR were verified by sequencing.

Cell Culture and Transfection—MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C and 5% CO_2. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stably expressing clones were selected with 1 mg/ml Geneticin. Sf9 cells were grown in suspension in Sf-900III serum-free medium (Invitrogen) at 27 °C with shaking at 130 rpm and transfected using the BaculoGold transfection kit (BD Biosciences) according to the manufacturer's instructions.

Zebrafish Care—Zebrafish were maintained under standard conditions as described previously (23). Embryos were maintained at 28.5 °C in egg water (24). All experiments were performed in strict accordance to standard guidelines for zebrafish work and approved by the Animal Institute Committee at Albert Einstein College of Medicine.

Protein Purification—Sf9 insect cells (200 ml at 2×10^6 cells/ml) were infected with high titer recombinant baculovirus. Cells were grown for 2 days before centrifugation and resuspension of cells in 30 ml of lysis buffer consisting of 50 mM sodium phosphate, pH 7.0, 500 mM NaCl, 1% Nonidet P-40 alternative (Calbiochem), and Complete EDTA-free protease inhibitor mixture (Roche Applied Science). Lysate was sonicated and centrifuged to remove cell debris. One milliliter of potato carboxypeptidase inhibitor-Sepharose resin, a generous gift from Prof. F. Xavier Avilés, was washed with lysis buffer before adding it to clarified lysate and incubating batchwise at room temperature for 30 min. Resin was transferred to a column for washing with lysis buffer followed by Nonidet P-40-free lysis buffer. CPO was eluted with 10–15 ml of elution buffer (100 mM Na_2CO_3, pH 11.2, 500 mM NaCl), dripping directly into 1 M sodium acetate, pH 5.0 to immediately neutralize the eluate. Protein concentration was measured by Bradford assay.

Carboxypeptidase Assays—The 3-(2-furyl)acryloyl (fa)-peptide substrates (Bachem) were dissolved in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl to a concentration of 0.5 mM. Enzymatic cleavage of substrates was measured by a decrease in absorbance at 340 nm at 25 °C. To determine enzyme pH optimum, substrate was dissolved in 50 mM Tris acetate buffer containing 150 mM NaCl at the indicated pH values. For kinetic constant determination, the initial reaction rate was determined using a range of substrate concentrations from 30 μM to 1 mM and

enzyme concentrations from 0.3–50 ng/μl, depending on the substrate, followed by nonlinear regression analysis using GraphPad Prism. All inhibitors were dissolved in water and preincubated with enzyme for 1 h prior to the addition of substrate (0.5 mM fa-EE at pH 7.5). Inhibition experiments and pH and substrate optimum experiments were performed with zCPO enzyme at a concentration of 0.4 ng/μl and hCPO enzyme at 4.0 ng/μl. Purified porcine tubulin was obtained from Cytoskeleton, Inc.

Western Blotting—Proteins were resolved by SDS-PAGE on 10 or 4–15% SDS-polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose. Western blotting was performed according to a standard protocol with the following antibodies: rabbit RP1-CPO, RP2-CPO, and RP3-CPO (Triple Point Biologics; 1:1000 dilution); α-tubulin (clone DM1A, Sigma; 1:5000 dilution); tyrosinated tubulin (mAb 1864, Millipore; 1:5000); detyrosinated (Glu-) tubulin (Ab 3201, Millipore; 1:500); Δ2 tubulin (Ab 3203, Millipore; 1:500); poly(Glu) (a kind gift from Dr. Martin Gorovsky; 1:1500), B3 polyglutamylated tubulin (Sigma; 1:2000); GT335 polyglutamylated tubulin (Enzo Life Sciences; 1:3000), and IRDye 800-conjugated secondary antibodies (Rockland; 1:3000 dilution). Images were obtained and quantified using the LiCor Odyssey system.

Characterization of CPO Post-translational Modifications—Separation of CPO into Triton X-114 (Sigma) detergent and aqueous phases as well as digestion with phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma) was performed as described (25). Peptide *N*-glycosidase F (New England Biolabs) digestions were performed according to the manufacturer's protocol. Edman degradation of purified human and zebrafish CPO was performed by the Protein Microanalytical Laboratory at the University of Pittsburgh (Dr. John Hempel, director).

Immunofluorescence—MDCK cells were cultured on chamber slides (Lab-Tek). 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% BSA, cells were immunostained for 1 h with rabbit RP3-CPO (Triple Point Biologics; 1:1000 dilution), mouse anti-EEA1 (BD Biosciences; 1:500 dilution), mouse anti-LAMP2 (Lifespan Biosciences; 1:500 dilution), and mouse anti-Na$^+$/K$^+$-ATPase α subunit (Affinity Bioreagents; 1:500 dilution) antibodies. The cells were washed three times with 0.2% Tween 20 in PBS and then incubated with Cy2- (1:100 dilution) or Cy3 (1:800 dilution)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h. After five washes with 0.2% Tween 20 in PBS, cells were mounted in a small amount of Prolong Gold antifade reagent with 4′,6′-diamidino-2-phenylidole (DAPI; Molecular Probes).

Immunohistochemistry—Frozen human ileum sections (Zyagen) were fixed with 4% paraformaldehyde for 20 min. After blocking in PBS containing 5% BSA and 0.5% Triton X-100 for 2 h at room temperature, sections were incubated with primary antibody RP1-CPO or RP3-CPO (Triple Point Biologics) diluted 1:500 in blocking solution for 20 h at 4 °C. To control for nonspecific binding, an antibody raised against a 10-residue peptide corresponding to a *Drosophila*-specific form of carboxypeptidase D was used; no homologous protein exists in the human non-redundant protein database. Sections were washed

several times with PBS containing 0.2% Triton X-100 and incubated with Cy3-conjugated anti-rabbit secondary antibody (1:800; Jackson ImmunoResearch Laboratories) for another 20 h at 4 °C. After washing, sections were mounted with Prolong Gold antifade reagent containing DAPI (Molecular Probes). Images were obtained on a Nikon Eclipse microscope supplemented with a cooled charge-coupled device camera (Roper Scientific).

RT-PCR—RNA was extracted from zebrafish embryos using the RNeasy minikit (Qiagen). RNA quality was assessed by formaldehyde gel electrophoresis. First strand cDNA synthesis was performed using the Superscript III First Strand Synthesis System (Invitrogen). Quantitative real time PCR was performed on an ABI 7900 using Power SYBR® Green PCR Master Mix (Applied Biosystems). Data were normalized to β-actin expression and shown as relative expression using the $2^{-\Delta\Delta CT}$ method.

In Situ Hybridization—A probe for *in situ* hybridization of CPO mRNA was generated by *in vitro* transcription from linearized pCRII-zCPO plasmid using a DIG (digoxigenin) RNA labeling kit (Roche Applied Science) with T3 or T7 RNA polymerase according to the manufacturer's protocol. The lithium chloride-precipitated RNA probe was dissolved in water, and quality was assessed by formaldehyde gel electrophoresis. Zebrafish embryos were fixed in 4% paraformaldehyde in PBS overnight at 4 °C and processed for *in situ* hybridization according to standard protocols (26) using a probe hybridization temperature of 70 °C. Staining reactions were performed with the alkaline phosphatase substrate BM purple (Roche Applied Science).

RESULTS

CPO Is a Conserved Enzyme with Unique Structure—The only published report on CPO describes a partial sequence that lacks the initiation methionine, a signal peptide, and most of the proregion (15). In the decade since this publication, several sequences for CPO have appeared in various internet databases, some of which contain an initiation methionine and signal peptide but not an extended prodomain that is expected to be required for enzyme folding based on related A/B subfamily peptidases. To explore this in more detail and to see whether the unique features of CPO were conserved, CPO orthologs from the genomes of a wide range of vertebrate species were identified (Fig. 2). CPO was present in most vertebrate species examined, including mammals, birds, and fish. Exceptions included the mouse and rat; only a pseudogene could be found in the mouse genome, and no ortholog was identified in the rat genome. All CPO orthologs exhibited strict conservation of critical active site residues necessary for zinc coordination (His-69, Glu-72, and His-196; bovine CPA numbering according to convention), catalysis (Glu-270), and C-terminal specificity (Arg-127and Arg-145; Fig. 2). In addition, residue 255, known to impart amino acid specificity within the CPA/B subfamily, is a conserved Arg in all CPO orthologs. This is a unique feature of CPO that predicts specificity for C-terminal acidic amino acids.

All orthologs of CPO contained an N-terminal signal peptide and a catalytic CP domain (Figs. 1*B* and 2). Mammalian orthologs contained a short ~20-amino acid N-terminal

FIGURE 2 (sequence alignment):

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                    <------- signal peptide ------->  <------------ N-terminal domain/prodomain ------------>
Human CPO      MKPLLETLYLLGMLVPGGLG--------------------------------------------------------------  20
Cow CPO        MKPLLGTFYLLGMLVPGWLG--------------------------------------------------------------  20
Dog CPO        MKPLLGTLCLLGILIPEGLG--------------------------------------------------------------  20
Rabbit CPO     MKTLLGTLYLLCMLIPGVLG--------------------------------------------------------------  20
Chicken CPO    MLLFLGMAFSLGILIPGKFS--------------------------------------------------------------  20
Finch CPO      MTHLWLSLGITFSLGILIPGNFG-----------------------------------------------------------  23
Zebrafish CPO  MMQASITSILVVLTLVAQDRLAGG----------------------------------------------------------  24
Stickleback CPO MLWPALLGLVALLSATSA---------------------------------------------------------------  18
Human CPA1     MRGLLVLSVLLGAVFGKEDFVG-HQVLRISVADEAQVQKVKELEDLEHLQLDFWRGPAHPGSPIDVRVPFPSIQ  73

                     <------------------------------------------------->
Human CPO      ----------YDRSLAQHRQEIVDKSVSPW----------S-LETYSYNIYHPMGETYEWMRETSEKYKEVVTQHF   75
Cow CPO        ----------YDRSLTQQRQEVVDKSVSPW----------SILETYSYNRYHPMGETYQWMSETREKYTEVVTQHF   76
Dog CPO        ----------YDGSLAQPRREVVEETGSPW----------QSLDTYSYTRYHPMEETYQWMIOTSEKYAGMVATQHF  76
Rabbit CPO     ----------YDRSLAQHGEEVVDKTVSPW----------RSLETYSYDKYHSMEETYQWMNOTSEKYTDVVTQHF   76
Chicken CPO    ------LKLQYDGILIHDVQELVDQSTP-DTSSQK-----QIPESYNYTQMHPMEETYTWMAOTQKNNSELVTQHF   84
Finch CPO      ------LKLQYDRVLIPDVQKLVDQSMPRERKSHR-----QVSGGYVYTEYHPMEETYQWMTOTQKNNSELVTQHF   88
Zebrafish CPO  ------------------------------------LEHKSYDYTKYHTVDETSAWWNOMQRENPDVVSTMT   60
Stickleback CPO ----------------------------------AVTERAEYDYFKYHPMPETSQWWDOVATDHPDVVTVMK   56
Human CPA1     AVKIFLESHGISYETMIEDVQSLLDEEQEQMFAFRSRAR---STDTENYATYHTLEETYDELDLLVAENPHLVSKIQ  147

                                                            H69  E72
Human CPO      LGVTYETHPMYYLKISQPSGNPKKIIWMDCGIHAREWIAPAFCOWFVKETLONHKDNSSLRKLLRNLDFYVLPVLNI  152
Cow CPO        LGMTYESRPMYYLKISQPSSNPKKIIWMDCGIHAREWIAPAFCOWFVKETLONYKDNSRLRRLLKNLDFYVLPVLNI  153
Dog CPO        LGMTYETRPMYYLKISQPSDNPKKIIWMDCGIHAREWIAPAFCOWFVKETLONYKDNPRLGRFLRNLDFYVLPVLNI  153
Rabbit CPO     LGMTYETRPMYYLKISQPSSNPKSIIWMDCGIHAREWIAPAFCOWFVKETLONYKDDLRISRCLRNLDFYVLPGLNI  153
Chicken CPO    LGETVENRTIYYLOISQPSDKTKKIIWMDCGIHAREWISPAFCOWFVKETLONYRSDPKIRKFLONMDLYLLPVLNI  161
Finch CPO      LGKTSENRTMYYLOISQPSNKTKKIIWMDCGIHAREWISPAFCOWFVKETLONYKTDPKISRFLONLDLYVLPVLNI  165
Zebrafish CPO  YGQTYEKRNTILLKIGFSSTTPKKAIWMDCGIHAREWIAPAFCOHFVKEVLGSYKTDSRVNMLFKNLDFYITPVLNM  137
Stickleback CPO YGQTYEKRTIRLLKIGLDTGEKKAIWMDCGIHAREWIAPAFCOYFVSOTLKAHKTDLKMQEMLKNMDFYVTPVLNM  133
Human CPA1     IGNTYEGRPIYVLK-FSTGGSKRPAIWIDTGIHSREWVTQASGVWFAKKITODYGQDAAFTAILDTLDIFLEIVTNP  223

                         R127                        R145
Human CPO      DGYIYTWT--TDRLWRKSRSP--HNNGTCFGTDINRNFNASWCSIGASRNCQDQTECGTGPVSEPETKAVASHIESK  225
Cow CPO        DGYIYTWT--TDRLWRKSRSS--HNNGTCFGTDINRNFDASWCSIGASHNCESLTECGTGPMSEPETKAVSSHIESK  226
Dog CPO        DGYIYTWT--TDRLWRKSRSS--HNNGTCFGTDINRNFNVSWCSIGASKNCQDLTICGSGPVSEPETKAVSSHIESK  226
Rabbit CPO     DGYIYSWI--TDRLWRKSRSL--HDNGTCFGTDINRNFNASWCSIGASKNCQDLTGCGTGPASEPETKAVSSHVESQ  226
Chicken CPO    DGYIYSWE--TARLWRKNRSP--HMNGTCYGTDINRNFNSSWGTLGVSYNCSSEIFCGTGPESEPETRAVVQLIERK  234
Finch CPO      DGYIYSWE--EDRLWRKSRSP--YENGTCYGTDINRNFNSSWGSVGVSFNCSSDVHCGSGPESEPETRAVAQFIKSK  238
Zebrafish CPO  DGYIYSWLNNSTRLWRKSRSPC-HENSTCSGTDINRNFYANWGMVGISRNCCSEVYNGATALSEPEAEAVTDHLGAH  213
Stickleback CPO DGYMYTWENNGTRLWRKNMSPG-PFGCNCTGVDINRNFDANWGTLGVSSNCCSEIYYGSRPVSEPEADAVTYFVGTR  209
Human CPA1     DGFAFTHS--TNRMWRKTRSHTAG--SLCIGVDPNRNWDAGFGGLSGASSNPCSETYHGKFANSEVEVKSIVDFVKDH  296

                    H196                                                     Y248      *255
Human CPO      KDDILCFLTMHSYGQLLLTPYGYTKNKSSNHPEMIOVGQKAANALKAKYGTNYRVGSSADILYASSGSSRDWARDIG  302
Cow CPO        KENIACFLTMHSYGQLLLVPYGYTKNKSNNHEELIOVGQKAANALKAKHGTNYRVGSSADILYATSGSSRDWARDIG  303
Dog CPO        KESIVCFLTMHSYGQYILLPYGYTKNKSSNHEELIOVGQKAANALKKKHGTNYRVGSSADILYSSSGSSRDWARDIG  303
Rabbit CPO     KENIVCFLTTHSYGQYILLPYGYSTKNKSSNHEELIOVGQKAANALKAKHGTNYRVGSSADILYSSSGSSRDWARDIG  303
Chicken CPO    KSDILCYLTTHSYGQYILLPYGSTTEPPSNNEELMRVAQTAAAALTEKYGTSYEVGPTSLITYSNSGSSRDWAHMIG  311
Finch CPO      KSDILCYLTTHSYGQLLLTPYGSTTKPPSNNEELMOVAKEAAAALTGKYGTSYRVGSTALILYSNSGSSRDWAHTIG  315
Zebrafish CPO  QNHLLCYLTTHSYGQLLLVPYGHPNISAPNYDELMEVGLAAAKAIKAVHGKSYKVGSSPDVILYPNSGSSRDEARLIG  290
Stickleback CPO PEEFLCFLTTHSYGQLLLVPYGHPNFTAYNYELMEVGLGAADAIRKVHGMNYTVGSSPDVILYPNSGSSRDWAHMQK  286
Human CPA1     GN-ILKAFISTHSYSQLLMYPYGYKTEPVPDQDELDOLSKAAVTALASLYGTKFNYGSIIKAIYQASGSTIDWTYSQG  372

                   E270                                     <------ C-terminal domain ------>
Human CPO      IPFSYTFELRDSGTYGEVLPEAOIOPTCEETMEAVLSVLDDVYAKHWHSDSAGRVTSAIMLLGLLVSCMSLL  374
Cow CPO        IPFSYTFELRDNGTYGEVLPETOIOATCEETMEAVLSVLDDVYEKYWYTNSARKAKSTALVLGLLMSFMSLL  375
Dog CPO        IPFSYTFELRDNGTYGEVLPEAAOIOATCEETMEAVLSVLDDVYEKYWNSDSAGKVTPATLVLGLLVSLVSIF  375
Rabbit CPO     IPFSYTFELRDNGTYGEVLPEAOIOPTCEETMEAVLSVLDDVYAKYWLSNSAGKVTSIITVVLSLLMSLIALL  375
Chicken CPO    IPFSYTFELRDKGTYGEVLPADOIOPTCEETMLAVTALLDYVDQKYHP-GGATMLTSGSLLGLSHCLTAVLTLIVT  385
Finch CPO      IPLSYTFELRDTGTHGEILPANOIOPTCEETMLAVTTLLDYVDKKYHP-NGHVVLTSSSLGLSLCLSIVLTWSVS  389
Zebrafish CPO  IPYSFTFELRDEGQHGEILPEDOIOPTCOEAYEGAMSLTNYVHDKNHK--NTAITVTAILWTTLMALWLTSTHVF  363
Stickleback CPO IPFSFTFELRDKGAFGEILPEDOIRPTCOEAYSGALHLITYAHDKTHS--GAVATAAAILLSITDLLAVGVTGNSLL  359
Human CPA1     ILKYSFTFELRDTGRYGFILPASOIIPTAKETWLALLTLMEHTLNHPY  419
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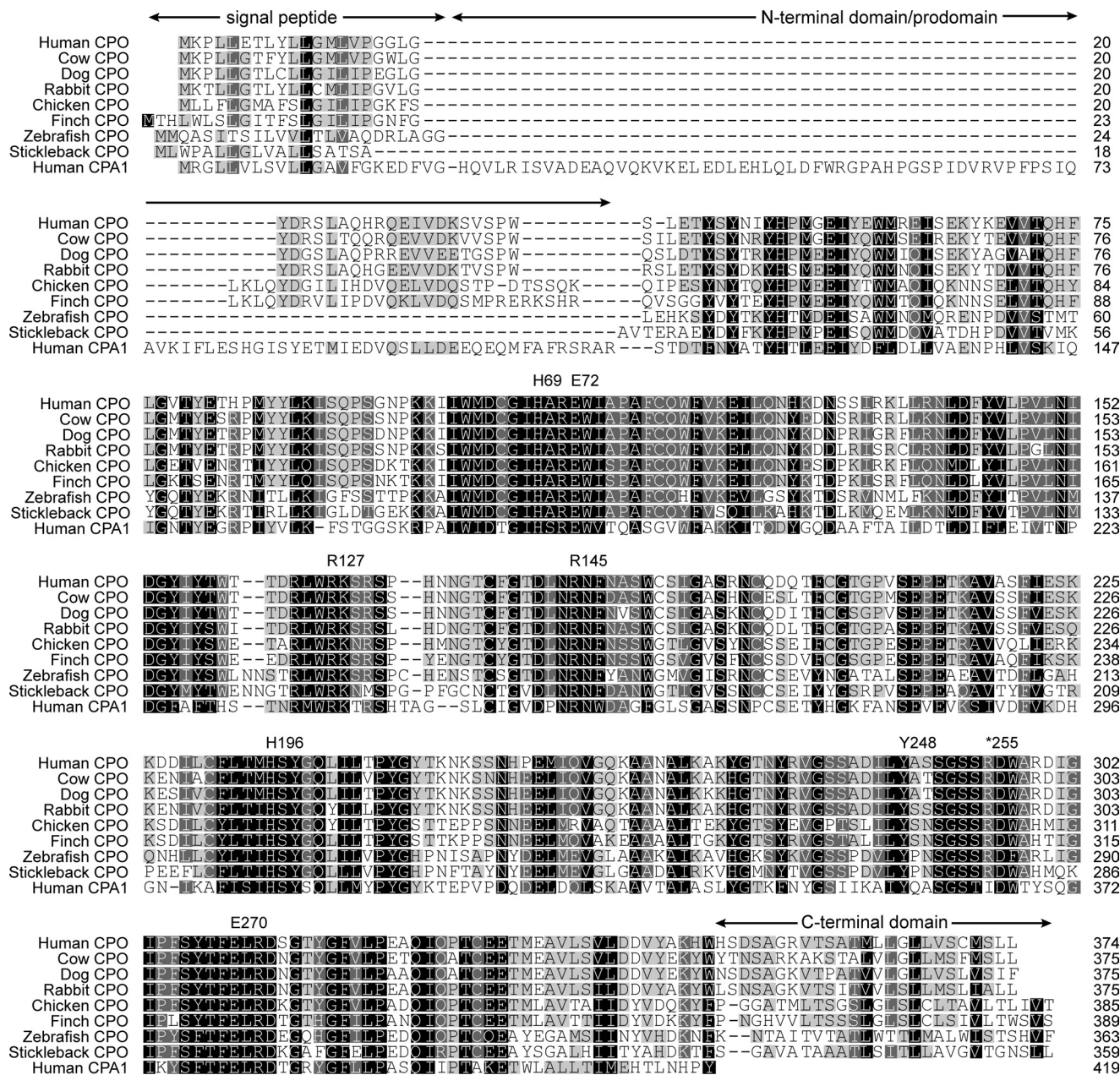
FIGURE 2. CPO protein sequences from representative species. Genomic and cDNA databases (Ensembl and NCBI) were searched for CPO sequences, which were aligned with each other and with human CPA1 using ClustalW. Signal peptide, N-terminal domain/prodomain, and C-terminal domain sequences are indicated as well as all critical active site residues necessary for zinc binding (His-69, Glu-72, and His-196), substrate binding (Arg-127, Arg-145, Tyr-248, and Arg/Ile-255), and catalytic activity (Glu-270; numbering system based on the position of residues in bovine CPA by convention). Greater sequence similarity is indicated by *darker shading*. Sequences shown are from the following database entries: human CPO, ENSP00000272852; cow CPO, ENS-BTAP00000025539; dog CPO, ENSCAFP00000019833; rabbit CPO, ENSOCUP00000008449; chicken CPO, gi|118093691:4613825–4669825; finch CPO, NW_002198919.1|Tgu7_WGA826_1; zebrafish CPO, NM_001145629; stickleback CPO, ENSGACP00000018671; and human CPA1, NP_001859.

domain with some homology to the equivalent portion of the CPA1 prodomain (Fig. 2). This N-terminal domain was slightly longer in birds (chicken and finch; 27–28 amino acids) and absent in fish (zebrafish and stickleback; Figs. 1*B* and 2). All CPO orthologs also contained a short C-terminal domain (25–27 amino acids in length) that was absent from all other members of the A/B subfamily (Figs. 1*B* and 2).

Purification of Human and Zebrafish CPO—Both human CPO and zebrafish CPO were expressed in Sf9 cells and purified using a potato carboxypeptidase inhibitor-Sepharose affinity chromatography approach. This one-step purification resulted in the complete purification of CPO, which was detected as a doublet by Coomassie or silver staining (supplemental Fig. S1, A and B). This doublet was also seen by Western blotting using an antibody to a C-terminal region of CPO (RP3-CPO) when CPO was transiently expressed in HEK293T cells (supplemental Fig. S1C) and stably expressed in MDCK cells (supplemental Fig. S1D). Additional Western blot analyses performed with Triple Point Biologics antibodies to the N-terminal regions of CPO (RP1-CPO and RP2-CPO) also showed doublets of the same size (not shown). None of these antibodies cross-reacted with zebrafish CPO.
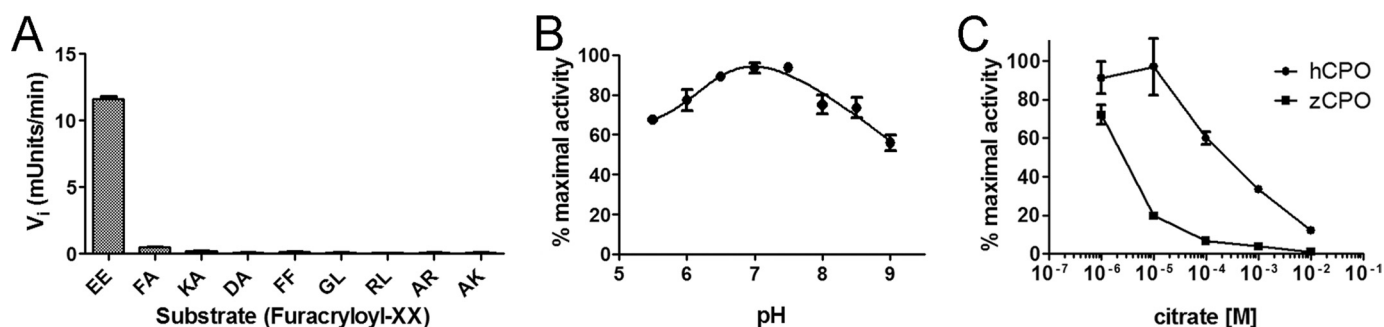
FIGURE 3. **Specificity and inhibition of CPO enzyme activity.** *A*, zebrafish CPO (0.4 ng/μl) was incubated with a variety of commercially available synthetic substrates at 0.5 mM concentrations. Reaction rates were determined by measuring the change in absorption at 340 nm. *B*, zebrafish CPO (0.4 ng/μl) was incubated with its optimal substrate, fa-EE, at a concentration of 0.5 mM and at a range of pH values to determine the pH optimum of CPO. *C*, citrate was found to inhibit the enzymatic activity of CPO assayed with 0.5 mM fa-EE, pH 7.5, although inhibition was less potent toward the human enzyme than the zebrafish enzyme. Human enzyme was incubated at a concentration of 4.0 ng/μl, whereas zebrafish CPO was incubated at 0.4 ng/μl. For all panels, *error bars* show S.E. for triplicate determinations.

To determine whether the CPO doublet was due to *N*-glycosylation, purified protein was digested with peptide *N*-glycosidase F. This digestion resulted in an increase in mobility of both human and zebrafish CPO, indicating that the protein was *N*-glycosylated ([supplemental Fig. S2](#)). Because CPO remained as a doublet after peptide *N*-glycosidase F treatment, *N*-linked glycosylation was not responsible for the difference between the two bands. Similar results were found when extracts from mammalian cells transfected with plasmids expressing CPO were treated with peptide *N*-glycosidase F (not shown).

The CPO doublet could also be a result of partial processing of either the N-terminal or C-terminal domains. To determine the N-terminal sequence, Edman degradation was performed. The N-terminal amino acid sequence of zebrafish CPO (LEHKS) and human CPO (YDRSL) both immediately follow the predicted signal peptide cleavage sites (see Fig. 2). No other N-terminal sequence was detected, indicating that the short N-terminal domain present in human CPO remains attached to the protein and is not cleaved like the prodomains of other CPs.

Enzymatic Characteristics of Purified Human and Zebrafish CPO—The enzymatic characteristics of purified CPO were determined. Zebrafish CPO was tested with a panel of commercially available CP substrates at pH 7.5 to determine its substrate specificity. Consistent with its predicted specificity for acidic amino acids, the substrate containing a C-terminal glutamate (fa-EE) was cleaved much more rapidly than substrates containing hydrophobic or basic C-terminal amino acids (Fig. 3*A*). In addition, the fa-EE substrate was used to assess the pH optimum for zebrafish CPO. The optimal pH for this enzyme was found to be 6.5–7.5, although CPO retained greater than 70% of maximal activity across a wide pH range from 5.5 to 8.5 (Fig. 3*B*).

Kinetic parameters were determined for both human and zebrafish CPO at pH 7.5 with the substrate fa-EE and three other substrates showing detectable cleavage (Table 1). Both human and zebrafish CPO exhibited greatest activity (K_{cat}) toward fa-EE followed by decreasing activity in the order fa-FA > fa-KA > fa-FF. This indicates that C-terminal acidic amino acids are the best substrates for CPO. However, CPO was also able to cleave C-terminal hydrophobic amino acids with small residues (alanine) being preferred over large residues (phenylalanine). Both human and zebrafish CPO exhibited K_m

TABLE 1
Kinetic constants for human and zebrafish CPO

Substrate	K_{cat} ±S.E.	K_m ±S.E.	K_{cat}/K_m ±S.E.
	s^{-1}	μm	$mm^{-1} s^{-1}$
Human CPO			
fa-EE	8.6± 0.5	325± 49	26.5± 7.5
fa-FF	0.27±0.02	284± 41	0.95±0.15
fa-FA	1.7± 0.2	549±148	3.1± 0.9
fa-KA	0.60±0.03	614± 71	0.98±0.12
Zebrafish CPO			
fa-EE	178± 10	346± 47	514± 75
fa-FF	1.6± 0.1	324± 37	4.9± 0.6
fa-FA	14.6± 1.2	794±114	18.4± 3.0
fa-KA	3.9± 0.2	743± 80	5.2± 0.6

values toward substrates with C-terminal alanine that were about double the K_m value toward C-terminal glutamate and phenylalanine. K_{cat}/K_m values obtained for zebrafish CPO were typically 5–20-fold greater than those obtained for human CPO, but the overall specificity of CPO appears similar for these two species.

CPO Is Inhibited by Common CP Inhibitors as Well as Citrate— A number of standard metallocarboxypeptidase inhibitors were incubated with CPO to determine their inhibitory activity (Table 2). Consistent with the acidic amino acid specificity of CPO, benzylsuccinic acid was a poor inhibitor of this enzyme. In contrast, potato carboxypeptidase inhibitor, which does not rely primarily on interactions with the C-terminal amino acid, was a potent inhibitor with an IC$_{50}$ of ~20–30 nM. Metal chelators EDTA and 1,10-phenanthroline were also effective inhibitors of CPO activity when used in the low millimolar range. A number of amino acids were tested to learn more about the specificity of this enzyme through product inhibition. Even at 10 mM concentrations, all amino acids tested resulted in relatively modest inhibition of CPO activity from 10 to 55%. However, as the best substrates and amino acid inhibitors of CPO were consistently dicarboxylic acids, we tested other compounds with multiple carboxylic acid groups. Succinate proved to be a modest inhibitor of CPO with an IC$_{50}$ in the low millimolar range. Citrate, which contains three carboxylic acid groups, virtually eliminated CPO activity at 10 mM. Further analysis of the inhibitory ability of citrate revealed an IC$_{50}$ of 3 μM toward zebrafish CPO and 200 μM for human CPO (Fig. 3*C*). No inhibitory activity was found for citrate concentrations of up to 1 mM toward bovine CPA1 (not shown), an enzyme

TABLE 2
Inhibition of human and zebrafish CPO activity

Inhibitor	Maximal activity ±S.E.	
	hCPO	zCPO
	%	
Benzylsuccinic acid		
0.1 mM	92.5±3.2	93.6±6.4
1 mM	87.8±3.7	65.6±7.6
Potato carboxypeptidase inhibitor		
10 nM	92.2±3.1	71.6±7.9
100 nM	15.5±1.3	7.6±0.6
EDTA		
1 mM	27.1±1.3	16.7±1.5
10 mM	4.3±0.4	9.1±1.3
1,10-Phenanthroline (1 mM)	10.0±0.4	35.7±2.3
Glycine (10 mM)	77.1±1.3	84.4±9.0
Asparagine (10 mM)	85.6±8.8	82.9±5.9
Glutamine (10 mM)	79.2±3.2	75.8±2.1
Aspartate (10 mM)	57.3±3.4	43.8±1.7
Glutamate (10 mM)	62.2±5.2	56.7±6.6
Succinate		
1 mM	68.7±5.6	60.1±4.0
10 mM	16.9±0.4	11.3±0.4
Citrate		
1 mM	33.5±1.9	3.8±0.4
10 mM	12.1±1.1	1.1±0.5

FIGURE 4. **CPO cleaves C-terminal glutamate from protein substrates.** Purified human and zebrafish CPO enzymes were incubated with 5 μg of purified porcine tubulin. Proteins were resolved by SDS-PAGE, and tubulin C-terminal and side-chain modifications were analyzed by Western blotting. All bands were quantified, normalized to total α-tubulin, and expressed as a percentage of tubulin not incubated with CPO enzyme. *B*, schematic showing the C terminus of α-tubulin and the antibodies that recognize the various modifications. *Tyr*, tyrosinated; *DeTyr*, detyrosinated.

with specificity for hydrophobic amino acids, suggesting that the inhibition of CPO was due to specific active site interactions unique to CPO. This is consistent with a recent report of the crystal structure of CPA1 in complex with citrate indicating a relatively weak competitive inhibition with a K_i of 5 mM (27).

CPO Can Cleave Large Proteins as Well as Peptides—In addition to the cleavage of small synthetic substrates by CPO, the ability of CPO to cleave larger proteins was investigated using tubulin as a model substrate. Tubulin normally undergoes a number of cleavages within the cytosol in which glutamates are removed both from the C terminus and from polyglutamates added to the γ-carboxyl group of glutamates near the C terminus. A number of antibodies are available that are specific for each of these modifications (Fig. 4*B*). Human and zebrafish CPO enzymes were incubated with purified porcine tubulin, which was then analyzed by Western blotting for C-terminal tubulin modifications. A decrease in signal was observed for most tubulin glutamate modifications even when tubulin was incubated with only 50 ng of CPO enzyme (Fig. 4*A*). In contrast, no large change was observed in the amount of tyrosinated tubulin, confirming that C-terminal tyrosine is not a good substrate for CPO. A dramatic increase in GT335 antibody signal was observed upon CPO incubation. The GT335 antibody is specific for the branch point glutamate formed through a γ linkage on a glutamate side chain (Fig. 4*B*) (28). It is most likely that long side-chain polyglutamates interfere sterically with the binding of the GT335 antibody to its epitope, the branch point glutamate. Removal of these interfering glutamates as seen with the poly(Glu) and B3 antibodies but not the branch point glutamate would result in a dramatic increase in the GT335 signal as seen upon incubation with CPO enzyme. It is interesting to note that CPO was able to decrease quantities of immunoreactive detyrosinated tubulin as well as $\Delta 2$ tubulin, likely making a $\Delta 3$ tubulin in which the C-terminal residue is a glycine. This

suggests that, unlike many related CPs, CPO readily cleaves substrates with glycine in the penultimate position.

CPO Is Membrane-bound via GPI Anchor—All CPO orthologs are predicted to have a functional N-terminal signal peptide directing them into the secretory pathway (Fig. 2). In addition, all CPO orthologs contain a C-terminal domain with a preponderance of hydrophobic residues. This C-terminal region fits the general consensus site for attachment of GPI. Utilizing the big-PI on-line program, CPO enzymes from mammals and chicken were predicted to be GPI-modified (Fig. 5*A*). CPO enzymes from finch, zebrafish, and stickleback fish were scored with lower probability for GPI modification, receiving scores that were just outside of the cutoff for likely modification. However, these scores were similar to that of carboxypeptidase M, which has been shown experimentally to be GPI-modified (22) despite the modest score from the big-PI program. Therefore, it is likely that CPO from most if not all species is GPI-modified.

To confirm that human CPO is GPI-anchored, it was stably expressed in MDCK cells, the cells were differentiated, and proteins were extracted into Triton X-114 buffer. This detergent enables the separation of detergent-soluble (membrane-bound) proteins from water-soluble proteins. Analysis of several different clones of stably transfected cells indicated that >90% of human CPO was present in the detergent phase and therefore likely to be membrane-bound (Fig. 5*B*). Detergent-soluble MDCK proteins were digested with PI-PLC, which is
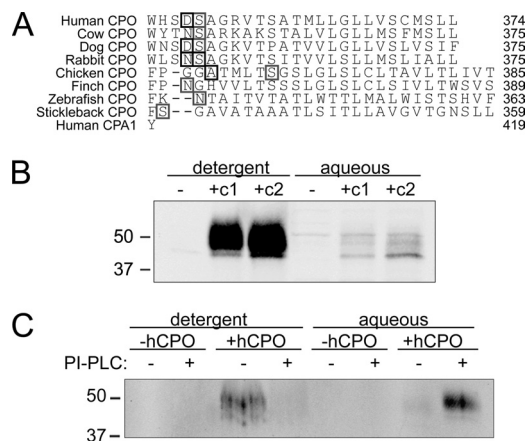
FIGURE 5. **CPO is GPI-anchored.** *A*, the most likely GPI modification sites predicted by the big-PI program are indicated with *black squares*. For mammalian CPO and chicken CPO, the program predicted a high likelihood of GPI modification ($p < 0.05$). For finch, zebrafish, and stickleback fish, the prediction was less confident ($p > 0.05$). *B*, proteins from two different clones (*c1* and *c2*) of differentiated MDCK cells stably transfected with human CPO (+) or empty vector (−) were extracted with Triton X-114, and detergent and aqueous phases were separated. CPO was found primarily in the detergent phase by Western blotting with a CPO-specific antibody. *C*, detergent-soluble proteins from control (−*hCPO*) or hCPO-expressing (+*hCPO*) MDCK cells were incubated with (+) or without (−) PI-PLC followed by a second phase extraction into Triton X114. Most CPO (detected by Western blotting) transitioned from the detergent to aqueous phase following incubation with PI-PLC, indicating the presence of GPI modification.

able to cleave GPI anchors, and re-extracted with Triton X-114. Upon PI-PLC cleavage, nearly all of the CPO transitioned from the detergent phase to the aqueous phase (Fig. 5*C*). This suggests that human CPO is present in cells as a membrane-bound GPI-anchored protein.

The membrane attachment of CPO was further investigated through immunofluorescence techniques. CPO was detected in MDCK cells stably expressing human CPO using two CPO-specific antibodies (RP1-CPO and RP3-CPO from Triple Point Biologics). Identical results were obtained for both antibodies. When cells were subconfluent, CPO was found in vesicular membranes (Fig. 6*A*, see *inset*) often arranged in a perinuclear fashion. Cells were costained for markers of early endosomes (EEA1) or late endosomes/lysosomes (LAMP2), but no colocalization with CPO was detected (Fig. 6*A*). Stably transfected MDCK cells were also allowed to grow as a monolayer for 6 days by which time they were differentiated into polarized epithelial cells. In these cells, CPO was found to colocalize with the Na$^+$/K$^+$-ATPase α subunit, a marker of the basolateral plasma membrane of differentiated epithelial cells (Fig. 6*B*).

CPO Is Expressed in Intestinal Epithelia—To determine the expression pattern of CPO in a variety of species, expressed sequence tag databases were searched. A number of CPO expressed sequence tags were identified from monkey, cow, sheep, pig, dog, chicken, and *Xenopus*, and most of these were isolated from intestinal tissues (Fig. 7*A*). The intestinal expression of CPO was confirmed by whole-mount *in situ* hybridization of 4-dpf zebrafish (Fig. 7*B*) at which time the intestines are beginning to develop (29). Sectioning of these embryos revealed that CPO mRNA was localized to the epithelial layer surrounding the intestinal lumen (Fig. 7*C*). To extend the analysis of zebrafish CPO expression beyond 4 dpf, quantitative real time

PCR was used. CPO mRNA was first detected in the zebrafish at 3 dpf and was strongly expressed by 5 dpf upon which levels decreased and remained at a steady level from 6 to 10 dpf (Fig. 7*D*). Expression of CPO was also detected by quantitative PCR in adult zebrafish intestinal tissue (not shown). The decrease of expression between 5 and 6 dpf when feeding is normally initiated suggested a possible regulation by feeding. However, no change in CPO mRNA levels was observed when fed and starved conditions were compared (Fig. 7*D*).

To determine the distribution of human CPO protein, frozen human tissue sections from the intestinal ileum were analyzed by immunohistochemistry. N-terminally directed (RP1) and C-terminally directed (RP3) antibodies to CPO were tested along with a control antibody. Both CPO antibodies showed a clear apical localization of CPO in enterocytes, the major epithelial cell type of the intestine (Fig. 7*E*). In many cases, CPO appeared to be concentrated on the surface of these cells as well (see Fig. 7*E*, *bottom right panel*). These results confirmed the expressed sequence tag data indicating intestinal CPO expression and showed the expression of CPO in intestinal epithelial cells to be conserved from zebrafish to human.

DISCUSSION

For many years, it has been accepted that CPA and CPB are the intestinal peptidases responsible for the production of amino acids from dietary proteins and peptides. This was thought to explain how amino acids were obtained from dietary sources. However, glutamate and aspartate are not good substrates for CPA and CPB but are known to be produced from dietary protein. In a study of protein digestion in the human intestine, Adibi and Mercer (30) found that free amino acids in the proximal jejunum largely matched the composition of the protein meal. However, upon reaching the proximal ileum, levels of free glutamate and aspartate greatly surpassed their expected levels based on the protein meal. This suggested that either absorption of these amino acids is low in the ileum or production is high. Our results suggest that CPO is present on the brush border of the ileal mucosa (and possibly other regions of the small intestine) where it is responsible for producing free glutamate and aspartate from dietary proteins. Interestingly, it has been shown that dietary glutamate is the single most important source of oxidative energy for intestinal enterocytes (31), suggesting an even more critical role for CPO in the intestine.

Unlike related members of the CPA/B subfamily of CPs, CPO does not appear to be regulated by propeptide cleavage. All other members of the A/B subfamily of metallocarboxypeptidases contain an N-terminal β-sheet-rich prodomain that must be proteolytically removed for full activity (32, 33). This zymogenic activation is important to restrict activity to where it is needed. Apparently, CPO is either always active or is regulated in other ways. There remains the possibility that mammalian and bird CPO is regulated through a very short prodomain as suggested by the relatively weak activity of human CPO. However, we have not found any evidence for cleavage of this domain.

In addition to its role in enzymatic inhibition, the N-terminal prodomain found in A/B CPs is also thought to function in protein folding (34). In fact, most CPs have domains other than
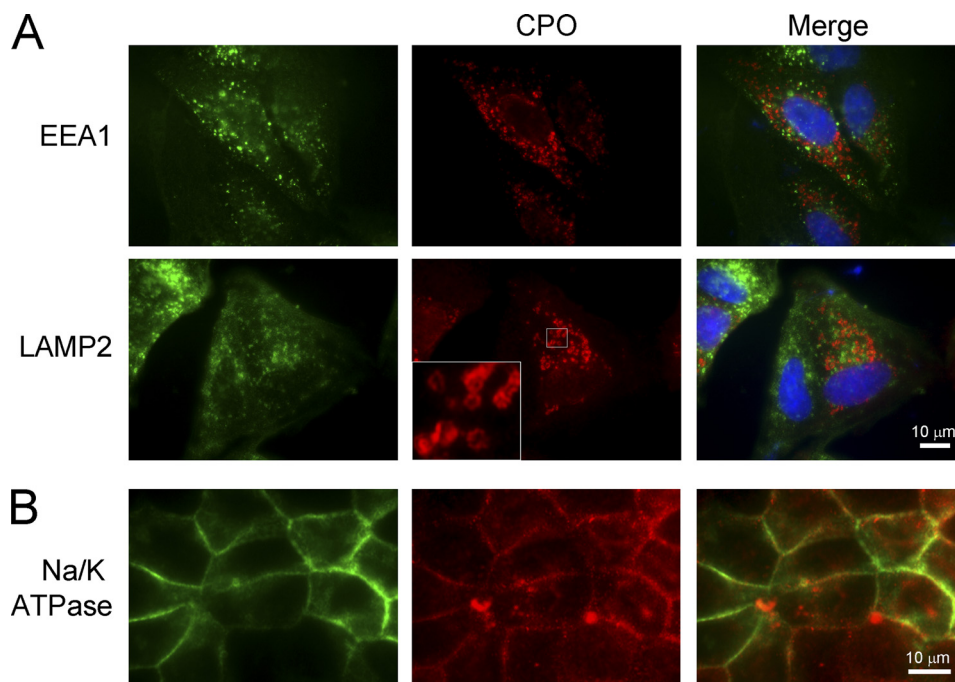
FIGURE 6. **Stably expressed CPO is localized to intracellular vesicular membranes as well as plasma membrane of MDCK cells.** *A*, MDCK cells stably expressing human CPO were fixed at a subconfluent state. CPO was identified by immunocytochemistry in vesicular structures (see *inset*). No colocalization of CPO was found with markers of either early (EEA1) or late (LAMP2) endosomes. Nuclei (*blue*) were stained with DAPI. *B*, when the above MDCK cells were differentiated into a polarized epithelial monolayer, CPO was more prominently seen at the plasma membrane as indicated by colocalization with Na^+/K^+-ATPase, a marker of the basolateral membrane. *Scale bars*, 10 μm.

the CP domain that are thought to function in folding: N/E members have a C-terminal β-sheet-rich transthyretin-like domain (35, 36), CCPs all have an N-terminal β-sheet-rich domain (16, 17), and the aminoacylases have a C-terminal β-sheet-rich domain (20). With the characterization of CPO presented here, it is now apparent that not all CPs require another large domain to fold and function properly. It is possible that another protein co-expressed with CPO performs this function, although expression of CPO in cells not normally expressing this protein resulted in active enzyme.

The selectivity of CPO for acidic amino acids is unique among the mammalian A/B subfamily of CPs and was predicted because of the presence of an Arg at the position equivalent to residue 255 in bovine CPA. Currently, there are five known mammalian CPs with acidic amino acid specificity. Aminoacylase 2 (also called aspartoacylase) functions in the deacetylation of *N*-acetyl-L-aspartate through a CP-like mechanism (20). This is in contrast to its close relative, aminoacylase 3, which preferentially deacetylates *N*-acetylated aromatic amino acids (37). Mutations in aminoacylase 2 cause Canavan disease, a fatal and progressive neurodegenerative disease, due to greatly elevated levels of *N*-acetyl-L-aspartate (38). Four members of the CCP subfamily have also been identified to have specificity for acidic amino acids (19, 39). Like aminoacylase 2, the CCPs are cytosolic enzymes (16). The CCPs have been proposed to play a role in removal of glutamate from the C termini of α-tubulin (19, 39). One of these enzymes, CCP1, is mutated in a classical mouse mutant, Purkinje cell degeneration (*pcd*), resulting in degeneration of Purkinje cells and several other neuronal cell types (40–42).

In addition to those mentioned above, several other CPs that cleave acidic amino acids have been identified in non-mamma-

lian species; like CPO, these other enzymes are members of the A/B subfamily of CPs. One of these enzymes was purified from the marine annelid *Sabellastarte magnifica* and was found to cleave both hydrophobic and acidic amino acids (43). Another enzyme was identified in the *Aedes aegypti* mosquito and was predicted to have acidic amino acid specificity (44). Finally, a glutamate-specific CP was identified and characterized from the insect pest corn earworm *Helicoverpa armigera* (45). Other non-M14 enzymes with acidic amino acid-specific carboxypeptidase activity exist, including glutamate carboxypeptidase II, an extracellular enzyme that hydrolyzes the neuropeptide *N*-acetylaspartylglutamate (46), and γ-glutamyl hydrolase, a lysosomal enzyme that hydrolyzes folate polyglutamates (47).

Many of the above mentioned CPs are known to play regulatory roles through their substrate cleavages. In addition to its digestive role, CPO might also regulate the activity of bioactive proteins and peptides. When expressed in MDCK cells, CPO was found localized to the basolateral membrane, suggesting a role in processing extracellular adhesion and signaling proteins. Although CPO appeared to be predominantly apical in human enterocytes, a role on the basolateral membrane or within the cell during vesicular trafficking cannot be ruled out. The activity of CPO is close to maximal even at the low pH values found within the secretory system, and because activation by removal of a prodomain is not necessary, the enzyme is likely to be fully active upon folding within the endoplasmic reticulum.

Although CPO was found largely in enterocytes, some colocalization was observed with chromogranin A within enteroendocrine cells (data not shown), further suggesting a potential role in intracellular peptide maturation and regulation. Several peptides that contain acidic C-terminal amino acids are pro-
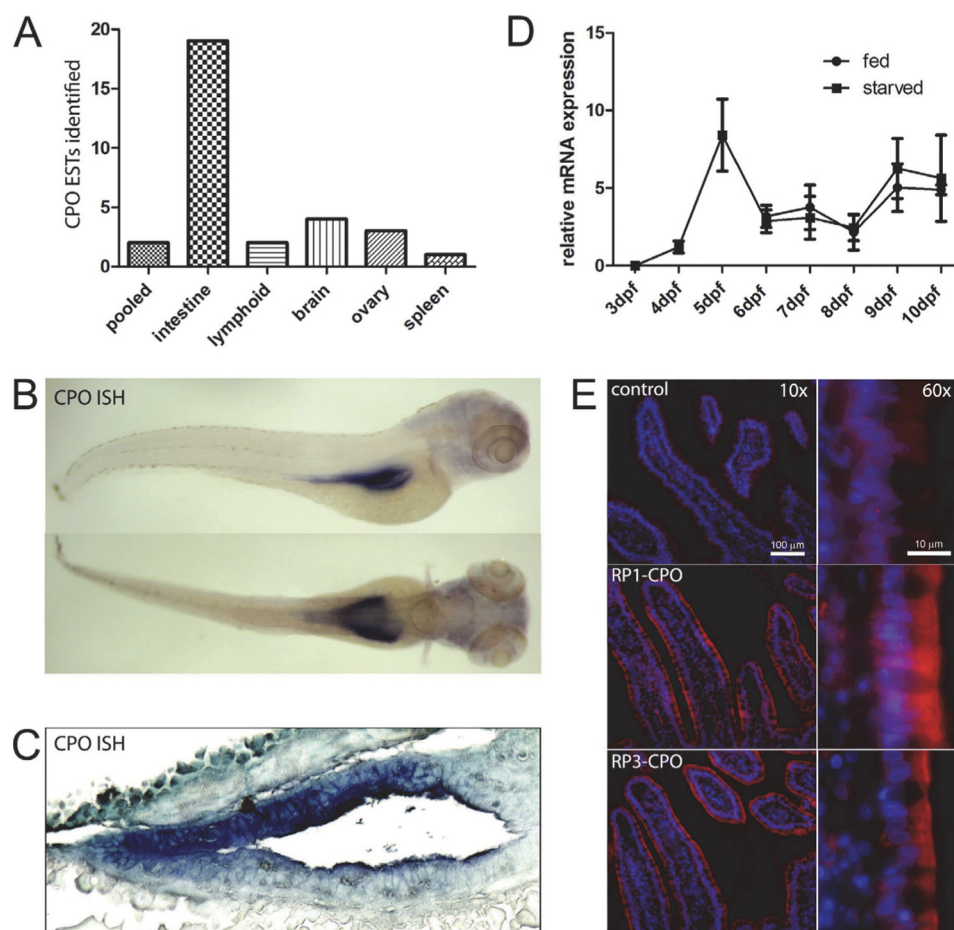
FIGURE 7. **CPO is expressed in intestinal epithelial cells.** *A*, a database search of expressed sequence tags (*ESTs*) for CPO mRNA from all species revealed 19 sequences from intestinal tissues and four or fewer from other tissues. *B*, *in situ* hybridization (*ISH*) of 4-dpf zebrafish indicated that CPO mRNA was found predominantly in intestinal tissues. Both lateral and dorsal views are shown. *C*, the above fish stained for CPO by *in situ* hybridization were paraffin-embedded, sectioned sagittally, and counterstained with methyl green. *Blue in situ* hybridization stain showed epithelial expression of CPO mRNA. *D*, CPO mRNA abundance was determined by quantitative PCR, which showed expression after 3 dpf in both fed and starved states. *Error bars* indicate S.E. for three biological replicates, each assayed in triplicate. *E*, frozen sections of human ileum were stained by immunohistochemical techniques with an unrelated control antibody and two different antibodies, one raised against the N-terminal region of CPO and the other raised against the C-terminal region of CPO (shown in "*red*"). Nuclei were stained with DAPI (*blue*). Both low (*left*) and high (*right*) magnification indicated CPO staining in the apical region of enterocytes. *Scale bars* in *E*, 100 μm (*left*) and 10 μm (*right*).

duced in intestinal cells. GLP-2 is a 33-residue proglucagon-derived peptide containing a C-terminal aspartate that is secreted from the L cells of the distal small intestine and colon (48). GLP-2 is responsible for the stimulation of growth of crypt cells in the intestinal jejunum and ileum (49) and has also been reported to play a role in gastric emptying (50) and intestinal epithelial barrier function (51). However, to our knowledge, a 32-residue GLP-2 lacking the C-terminal aspartate has not been reported. Chromogranin A is a well known marker of enteroendocrine cells of the intestine (52) that produces a number of peptides having acidic C-terminal amino acids (53). Another well known intestinal proprotein, procholecystokinin, is also known to produce peptides with acidic C termini (53). However, the exact functions of these peptides are not known.

In conclusion, we have characterized the activity of a unique metallocarboxypeptidase, CPO, which is translated and folded as an active enzyme without the need for a typical prodomain. CPO has substrate selectivity for C-terminal acidic amino acids, is expressed predominantly in the epithelial cells of the intestine, and is GPI-anchored and enzymatically active in the extracellular space where it presumably processes dietary protein and peptides. The high degree of conservation of CPO in most vertebrates from fish to humans argues for an important function, although the absence of CPO in rat and mouse suggests it is not essential for life.

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