Sequence of human carboxypeptidase D reveals it to be a member of the regulatory carboxypeptidase family with three tandem active site domains

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We have cloned the cDNA for human carboxypeptidase D (CPD), a new B-type metallocarboxypeptidase that is membrane bound and has an acidic pH optimum. The 5.8 kb of cDNA sequenced contains an open reading frame of 4131 bp encoding 1377 amino acid residues. The sequence is similar (75 % identity) to duck gp180, a protein that was isolated, cloned and sequenced as a hepatitis B virus-binding protein but not characterized as a carboxypeptidase. Hydropathic analysis revealed a hydrophobic region at the N-terminus, representing the signal peptide, and one near the C-terminus that probably represents the transmembrane anchor. The most striking feature is the presence of three tandem carboxypeptidase homology domains that have sequence similarity to the regulatory B-type carboxypeptidase family, typified by carboxypeptidases M, E and N. Because of the three repeats, CPD is about three times larger (175–180 kDa) than other members of this family (approx. 50–62 kDa). Domain 2 is most closely related to carboxypeptidases M, E and N (45–48 % identity), followed by domain 1 (37–38 %) and domain 3 (20–27 %). There is much higher sequence identity in regions containing putative active site residues, and all catalytically important residues are strictly conserved in domains 1 and 2. In domain 3, however, only 1 of 8 active site residues is conserved, indicating that this portion might not be catalytically active. Northern blotting of mRNA from human tissues and cells showed high levels of CPD mRNA in placenta, pancreas and Hep G2 hepatoma cells, and smaller amounts in skeletal muscle, heart and HT-29 colon carcinoma and melanoma cell lines.

INTRODUCTION

Mammalian regulatory B-type carboxypeptidases comprise a subfamily of metallocarboxypeptidases that have higher sequence identity with each other (approx. 35–50 %) than with the pancreatic carboxypeptidase family (15–20 %) [1,2]. These enzymes perform a variety of important cellular functions including prohormone processing, regulation of peptide hormone activity, alteration of protein–protein or protein–cell interactions and transcriptional regulation [1,2]. A newly described member of this family, carboxypeptidase D (CPD), was identified in rat tissues [3] as well as human and mouse cells [4], purified to homogeneity from bovine pituitary [3], and partly purified from human placenta [4]. It was suggested [3] that bovine CPD might be a mammalian homologue of duck gp180, a protein originally identified as a duck hepatitis B virus-binding protein but not characterized as a carboxypeptidase [5]. This was based on the identity of seven out of ten residues at the N-terminus of CPD and the similarities in molecular mass. [3]. We found that antibodies raised against a C-terminal recombinant fragment of human gp180 immunoprecipitated the CPD activity from human and mouse cells and reacted with a 175 kDa protein in Western blots [4].

CPD is found in a variety of cells and tissues, including cell lines such as mouse J774A.1 monocyte/macrophages and AtT20 pituitary cells and human fibroblasts [4], and is relatively high in rat brain, adrenal, lung and kidney [6]. The enzyme has some similarities to carboxypeptidase E (CPE), such as a low pH optimum, but is much larger (175–180 kDa) and is membrane-bound [3,4]. We report here the cloning and sequencing of cDNA containing the entire coding region of human CPD and the distribution of its mRNA in human tissues and cells. On the basis of the sequence obtained, CPD is the human homologue of duck gp180 and contains three tandem carboxypeptidase-like domains in its 1377-residue sequence.

MATERIALS AND METHODS

Identification and cloning of the cDNA for human CPD/gp180

The amino acid sequence of duck gp180 was used to screen the GenBank database of expressed sequence tags using the TBLASTN program on the National Center for Biotechnology Information's BLAST (Basic Local Alignment Search Tool) server via the World Wide Web. Several human cDNA clones were identified whose partial sequences (250–400 bp) were highly similar (more than 70 % sequence identity) to duck gp180. Five clones with the following GenBank accession numbers were obtained from the I.M.A.G.E. (Integrated Molecular Analysis of Genome Expression) Consortium: H04765, H04861 and R68612, isolated from a human placental library; H21058 and R52214, from a human infant brain library. Initial sequencing and

Abbreviations used: CPD, carboxypeptidase D; CPE, carboxypeptidase E; I.M.A.G.E., Integrated Molecular Analysis of Genome Expression; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U65090.

restriction mapping of the clones revealed that three mapped to the N-terminal region of gp180; H04765 and H04861 were identical but cloned in the opposite orientation and R68612 overlapped the sequence of H04765 but was longer. H21058 and R52214 mapped to the C-terminal region and overlapped each other, but R52214 was the longest. After the initial analysis, clones H04765, R68612 and R52214 were chosen for further sequencing.

To obtain the putative missing 2.5 kb of cDNA from the middle of the sequence, two approaches were used: PCR amplification and cDNA library screening. PCR was performed with human liver cDNA (Clontech) and two primers designed from the sequences of clones R68612 and R52214: sense primer S1, 5′-GAACTGTCTTGTTGCAAGTACCCACC-3′ (corresponds to bp 1074–1099 in the final sequence) and anti-sense primer C1, 5′-GTATACTGTGATTTCCGGACAATGGCC-3′ (corresponds to bp 3543–3569 in the final sequence). The amplification was done with the Elongase system (Gibco/BRL), an enzyme mixture containing a DNA polymerase with 3′–5′ exonuclease activity for high-fidelity PCR. The reaction was performed with 300 ng of each primer and 200 ng of cDNA template as described by the manufacturer. A cDNA of the expected size, 2.5 kb (named PCR25), was amplified and cloned into a pCR3 vector with the TA cloning kit (Invitrogen). Sequencing of the cDNA product gave correct primer sequences and a sequence highly similar to that of duck gp180, indicating that it was the desired product.

cDNA library screening was performed on a macrophage-derived cDNA library [7] that was constructed in a Lambda ZAP II vector (Stratagene, La Jolla, CA, U.S.A.). Filters were hybridized with a ^{32}P-labelled cDNA probe from the insert of clone R68612 by using standard procedures [8]. Plaques of interest were subjected to a second round of screening and Lambda ZAP II phages were converted into a pBluescript SK plasmid vector by the *in vivo* excision protocol (Stratagene). Library screening yielded a 1.3 kb clone (called CPDMAK-01) that mapped to the middle region of duck gp180, overlapping the 2.5 kb PCR clone; this was used for further sequence analysis.

The 5′ end of the cDNA was obtained by screening a human placental 5′-STRETCH Plus cDNA library (Clontech) with the 5′ *Hin*dIII/*Bam*HI fragment of clone H04765; 40 positive clones were obtained and inserts from 12 of the clones were isolated and subcloned into a pGEM 7Z(−) vector. A 1823 bp *Eco*RI fragment (CPD-5′) containing the entire 5′ coding region of human CPD and overlapping with clones H04765, R68612, CPDMAK-01 and PCR25 was identified through restriction mapping and sequencing.

DNA sequence analysis

cDNA sequences were determined by a combination of manual and automated sequencing with both vector and specific primers, restriction fragments and nested deletions. Unidirectional deletions in the 2.5 kb PCR25 cDNA clone were constructed with the Nested Deletion Kit (Pharmacia Biotech., Uppsala, Sweden). Subclones generated in steps of approx. 200 nt were used for sequence analysis. For sequencing of the R52214 clone, the two *Hin*dIII-restriction fragments of the clone were subcloned into pBluescript. Manual sequencing was done with the dideoxy chain-termination method, employing [^{35}S]dATP and Sequenase (U.S. Biochemical Corp.). Automated cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions; sequences were analysed on an Applied Biosystems DNA Sequencing System (model 373A). The overall sequence was determined completely in both directions and all parts of the coding region were sequenced a total of at least three and up to six times. Carboxypeptidase sequences were compared by using the ALIGN program on the EERIE (École pour les Études et la Recherche en Informatique et Électronique) server via the World Wide Web.

Northern analysis

Total RNA was isolated from different cell lines by the guanidine thiocyanate/acid phenol method and separated and transferred to nylon membranes (Magna NT; MSI, Westbrough, MA, U.S.A.) as described [9]. For determination of mRNA levels in human tissues, a multiple-tissue Northern blot (Clontech Laboratories) was used. Membranes were probed with the ^{32}P-labelled 800 bp insert of clone R68612 or with a specific oligonucleotide probe (5′-ACGGTATCTGATCGTCTTCGAACC-3′) to 18 S RNA (as a control) with previously described techniques [10]. The blot was exposed to X-ray film for 1 week at −70 °C and then developed. The multiple-tissue Northern blot was scanned with a P.D.I. (Protein Design Institute) Discovery Series imaging densitometer to quantify the relative levels of CPD mRNA in each tissue. The peak area for each band was normalized to that of the 18 S RNA band in the same lane.

RESULTS AND DISCUSSION

An initial CPD sequence composed of 5138 bp was constructed from a combination of cDNA clones obtained from the I.M.A.G.E. Consortium, cDNA library screening and PCR amplification of human liver cDNA. However, the sequence still lacked part of the coding region 5′ to the *Not*I site at bp 664 (Figure 1). Numerous unsuccessful attempts were made to obtain the additional 5′ sequence by a variety of 5′ rapid amplification of cDNA ends (RACE) procedures with different primers and by screening several different human cDNA libraries with a labelled 5′ clone. Finally, screening a human placental 5′-STRETCH Plus cDNA library yielded a 1.8 kb clone (CPD-5′) containing the complete 5′ portion of the cDNA (Figure 1). The sequence in the 5′ 700 bp is very GC-rich (approx. 72%), explaining the difficulty in reverse transcription in the 5′ RACE attempts and its absence from normal cDNA libraries. The presence of five *Not*I sites in this 700 bp region is the probable reason for there being no clones with sequences containing this region in the database of expressed sequence tags. This is because the libraries used by the I.M.A.G.E. Consortium were constructed by priming first-strand cDNA synthesis with a synthetic *Not*I-oligo(dT) primer followed by the ligation of double-stranded cDNA to *Eco*RI adaptors, digestion with *Not*I and then cloning into the *Not*I and *Eco*RI sites of a modified pT7T3 vector.

The total 5801 bp of cDNA thus obtained contains an open reading frame of 4131 bp encoding 1377 amino acid residues (Figures 1 and 2). Although it does not contain the classic AATAAA polyadenylation signal, there is a potential alternative polyadenylation signal (TATAAA) starting 26 bp upstream of a 28 bp poly(A) tail at the 3′ end of the sequence (Figure 2). Hydropathic analysis indicated the presence of two significantly hydrophobic regions, one at the N-terminus and one near the C-terminus (Figure 3). The N-terminal sequence (−30 to −1; Figure 2) has the characteristics of a typical signal peptide, with a consensus signal peptidase cleavage site that would result in an N-terminal sequence (AHIKKAEA…) matching that determined for purified bovine CPD [6]. Human CPD is a membrane protein that requires detergent for solubilization [4]. Apart from the N-terminal signal peptide, a stretch of 26 residues near the C-

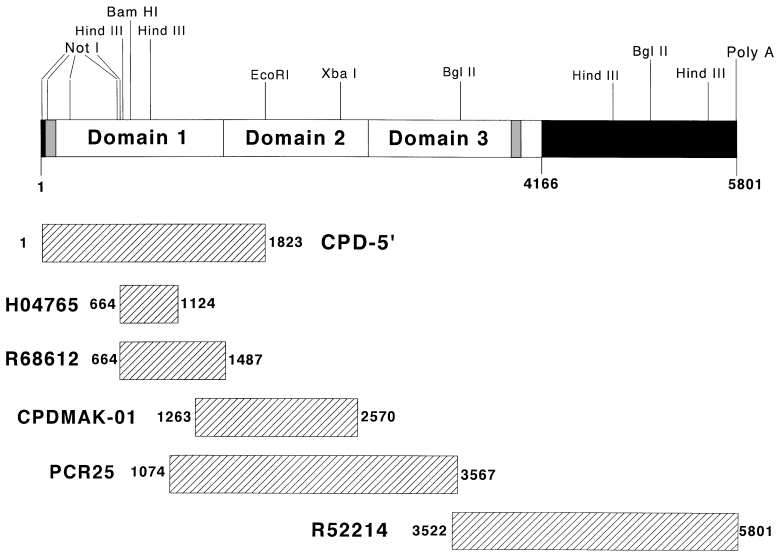

Figure 1 Cloning of human CPD

The 5801 bp sequence of human CPD was constructed from the sequences of six clones (hatched bars), which are numbered and positioned in accordance with their location in the overall sequence. For the sources of the clones see the Materials and methods section. A schematic diagram of the composite sequence is shown at the top, with restriction sites marked. The open bar represents the coding sequence, the black bars are the 3′ and 5′ untranslated regions and the grey boxes mark the hydrophobic sequences. The three tandem carboxypeptidase-like domains are numbered 1 to 3 and their positions marked accordingly. For further details see the text.

terminus (1267–1292) is the only hydrophobic sequence long enough to form a transmembrane anchor (Figures 1 and 2). The deduced amino acid sequence also contains 16 potential Asn-linked glycosylation sites (Asn-Xaa-Ser/Thr) (Figure 2). Of these, it is unlikely that the site at Asn^{184}-Arg^{185}-Ser^{186} is used (not underlined in Figure 2) because Asn^{184} and Arg^{185} are two potential active site residues involved in substrate binding (see below).

The most striking feature of the human CPD sequence is the presence of three tandem carboxypeptidase homology domains, here named 1, 2 and 3 (from N-terminus to C-terminus) (Figure 1). These domains (CPD-1, CPD-2 and CPD-3) have the highest sequence similarity (20–48 %) to the regulatory B-type carboxypeptidase family, typified by carboxypeptidases M, E and N (Table 1). Because of the three tandem repeats, CPD is about three times larger (175–180 kDa) than other members of this family (approx. 50–62 kDa). CPD-2 is most closely related to carboxypeptidases M, E and N, with 45–48 % sequence identity. The lowest identity (31 %) is with the carboxypeptidase homology domain of mouse AEBP1, a transcriptional repressor [11]. The sequence identity with human CPD-1 is somewhat lower: 37–38 % with CPs M, E and N, and 25 % with AEBP1; their similarity to CPD-3 is the lowest (20–27 %) (Table 1). Interestingly, the sequence identity of each domain with other members of this family is similar to that found when the three domains are compared with each other (Table 1). Thus CPD-1 and CPD-2 are most similar to each other (40 %), whereas CPD-3 has a lower identity with CPD-1 and CPD-2 (29 % and 30 %).

When the sequences of putative active site regions of regulatory carboxypeptidases [2] (i.e. containing zinc-binding and substrate-binding residues and the catalytic glutamic acid residue) are compared, the sequence similarity is much higher to CPD-1 and CPD-2 and the other regulatory carboxypeptidases (Figure 4). Here, out of a total of 77 amino acid residues, CPD-2 has 70 % identity with CPM, 74 % with CPN and 79 % with CPE, and CPD-1 has 74 %, 68 % and 68 % identity respectively. In these regions, CPD-1 and CPD-2 have 68 % sequence identity with each other. All of the putative active residues are strictly conserved between these enzymes (Figure 4). In contrast, the sequence identity in the active site regions of CPD-3 with CPD-1, CPD-2 and the other members of the family is much lower, ranging from 39 to 46 % (Figure 4). In addition, of the eight putative active site residues identified in the other CPs, only one is conserved in CPD-3 (Figure 4). Thus it seems unlikely that CPD-3 is an active metallocarboxypeptidase. Interestingly, in the transcriptional repressor protein, AEBP1, which has been reported to have carboxypeptidase activity [11], overall sequence identity in these regions with other members of the regulatory carboxypeptidase family (including CPD) is similarly low (36–52 %) and only two of the eight active site residues are conserved (Figure 4). In the position of the catalytic glutamic acid residue in other CPs, AEBP1 has tyrosine, and the sequence preceding this residue, Leu-Ser-Val-Tyr, bears a striking resemblance to that in CPD-3, Ile-Thr-Val-Tyr. Whether this means that AEBP1 and CPD-3 have an unusual but functional carboxypeptidase active site will require further study. Thus, on the basis of sequence similarity and conservation of active site residues, it is possible that CPD contains at least two functional active site domains, similar to that of the angiotensin I-converting enzyme, which contains two homologous, but non-identical, active sites [12].

The predicted protein sequence has relatively high identity (75 % overall) with that of duck gp180 (Figure 5). Duck gp180 was originally identified as a hepatitis B virus-binding protein and although cloning and sequencing revealed its similarity to other metallocarboxypeptidases, it has never been characterized as a carboxypeptidase [5]. The region designated domain 1 (residues −30 to 464 in the human sequence) is the least conserved, being only 64 % identical between the human and duck, whereas the rest of the sequence comprising domains 2 and 3 is 82 % identical (Figure 5). This is the consequence of three factors. First, the signal peptide is not well conserved between the

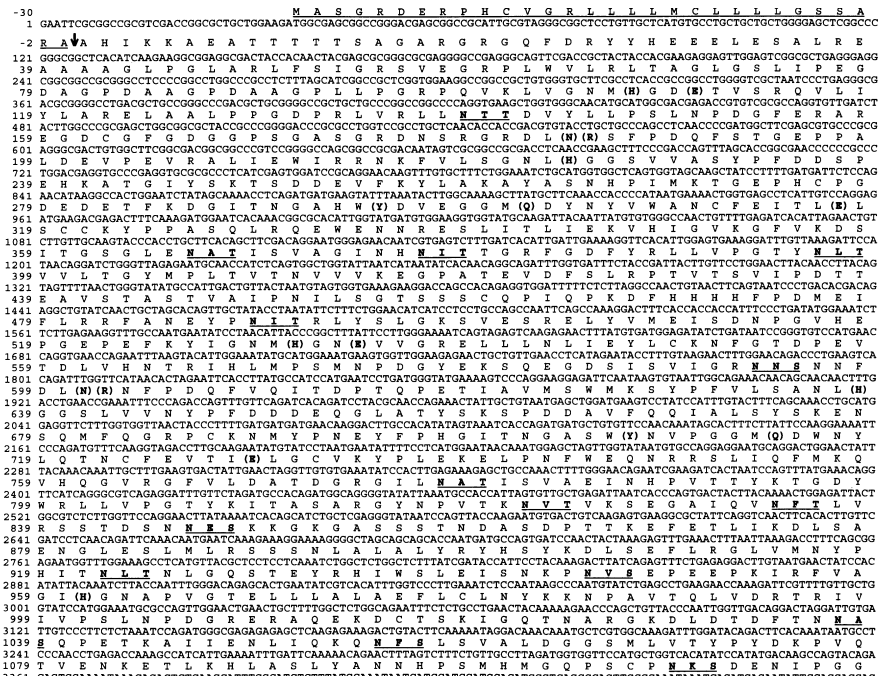

Figure 2 Sequence of human CPD

The cDNA sequence of human CPD and the deduced amino acid sequence are shown. Numbering of the residues is based on the putative signal peptidase cleavage site (arrow); the signal peptide (underlined) starts at −30 and the mature sequence at residue 1. Important residues are shown in bold type and are further marked as follows: putative active site residues that are conserved in other carboxypeptidases are in parentheses, potential Asn-linked glycosylation sites are singly underlined and the putative hydrophobic membrane-spanning sequence is doubly underlined. The underlined cDNA sequence near the end represents the potential polyadenylation signal.

two species (9 out of 30 residues). Secondly, the duck sequence contains three highly repetitive sequences that are not found in the human sequence, namely A^{9}AAGGGGGVGG^{19}, K^{80}KKKEEEEEEEEEEGEEGGGG^{100} and G^{172}GGGGGG-GEGGE^{184}. Thirdly, in a stretch of 34 residues at the C-terminus of domain 1 (431–464 in the human), only eight residues are conserved and there are six gaps in the human sequence (Figure 5). This is unlikely to be due to a sequencing error because the sequence in this region is clear and was confirmed in three separate clones that were sequenced a total of six times. This region probably constitutes a 'connecting peptide', linking domain 1 to domain 2, and as such might not have an important structural role that requires a high level of conservation. Similarly, there is a sequence of 29 residues at the C-terminus of domain 2 (838–866 in human) with only 11 conserved residues and four gaps in the duck sequence that might be the connecting peptide between domains 2 and 3 (Figure 5). Overall the duck sequence is 12 residues longer than the human, and 18 residues longer if only the mature protein (without the signal peptide) is considered. This is primarily due to the repetitive sequences in duck gp180 domain 1 and additional residues in the connecting peptide between domains 1 and 2 (Figure 5).

In domains 1 and 2, putative active site residues are strictly conserved between duck and human, and sequences immediately surrounding those residues are highly conserved between the two species (91% in domain 1 and 97% in domain 2) (Figure 5). Interestingly, even though domain 3 lacks most of the essential active site residues identified in other carboxypeptidases, the residues substituted in those positions in domain 3 are strictly conserved between duck and human, and the overall sequence identity in residues surrounding those regions is high (88%) (Figure 5). This indicates that domain 3 is not just an evolutionary remnant of a carboxypeptidase of no importance, but might have evolved to perform some important function, e.g. binding to one or more other proteins or to perform an unusual catalytic process.

Human CPD contains 15 potential glycosylation sites and duck gp180 contains 12, not counting the potential glycosylation site at the putative active site Asn (residue 197 in the duck and 184 in the human). Of these, the positions of 11 of the potential

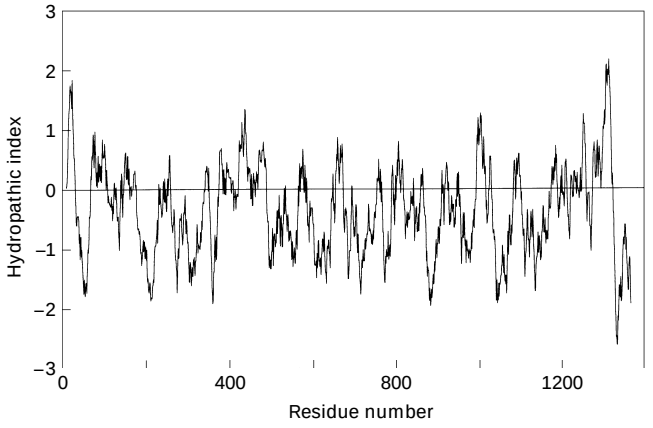

Figure 3 Hydropathic analysis of the deduced human CPD sequence

The hydrophobicity of CPD was determined by the method of Kyte and Doolittle [16] with a 19-residue window. Tracings above the centre line indicate hydrophobic regions, and below the line hydrophilic regions.

Table 1 Sequence identities between mammalian regulatory B-type carboxypeptidases

The percentage identities were calculated for the amino acid sequences of the mature enzymes (lacking the signal and propeptides). For AEBP1 the N-terminal 140 residues containing the discoidin-like domain were not included in the comparison. Abbreviations: h, human; m, mouse; CP, carboxypeptidase; h-CPD-1, h-CPD-2 and h-CPD-3, the three tandem carboxypeptidase repeat domains in the human CPD sequence; AEBP1, transcriptional repressor that binds to the AE-1 site of the *aP2* gene in preadipocytes.

	Identity (%)					
	h-CPN	h-CPE	h-CPD-1	h-CPD-2	h-CPD-3	m-AEBP1
h-CPM	41	43	37	45	27	27
h-CPN	–	49	38	45	25	36
h-CPE		–	37	48	20	33
h-CPD-1			–	40	29	25
h-CPD-2				–	30	31
h-CPD-3					–	25

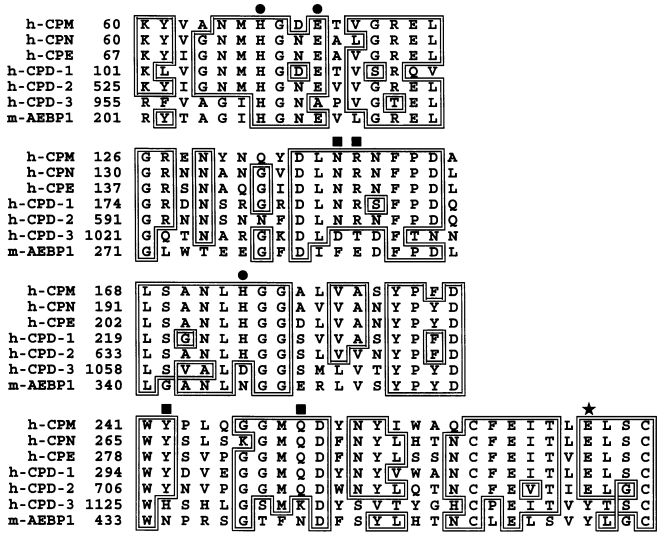

Figure 4 Comparison of the sequences around active site residues in mammalian regulatory B-type carboxypeptidases

Residues conserved in at least four of the sequences are boxed. Putative active site residues are marked as follows: ●, zinc-binding residues; ■, substrate-binding residues; ★, catalytic glutamic acid residue. Abbreviations: h, human; CP, carboxypeptidase; CPD-1, CPD-2 and CPD-3, the three tandem carboxypeptidase repeat domains in the CPD sequence; m, mouse; AEBP1, a transcriptional repressor protein with sequence similarity to carboxypeptidases that binds to the AE-1 site of the *aP2* gene (see [11]).

glycosylation sites are conserved between duck and human (Figure 5). The putative membrane-spanning sequence of 26 hydrophobic residues is highly conserved between duck and human, with only four conservative changes (Figure 5). Another sequence that is highly conserved is the presumed cytoplasmic domain C-terminal to the trans-membrane-spanning region where, out of 55 residues, only three changes are found in the human sequence (95% identity). This might indicate an important role for this region, for example in mediating intracellular sorting, protein–protein interactions or endocytosis. Indeed, the cytoplasmic domain contains several potential phosphorylation sites, two acidic regions (D^{1309}EYEDE^{1314} and D^{1333}ETDTEEE^{1340}) and a dileucine motif ($L^{1326}L^{1327}$) that could serve as a signal for endocytosis.

When human tissue mRNA was analysed by Northern blotting with a specific human CPD probe, three major bands of 7.4, 6.6 and 5.5 kb were seen in pancreas, placenta, heart and skeletal muscle (Figure 6). A very faint band of 4.2 kb was also visible in the placental mRNA lane but not in any other sample. Quantification by scanning densitometry revealed that the intensities of the 6.6 and 5.5 kb bands were similar and approx. 1.3–2.3-fold lower than the 7.4 kb band, which was the major form in all cases. The relative levels of CPD mRNA are highest in placenta and pancreas and approx. 3–4-fold lower in skeletal muscle or heart when normalized to the 18 S RNA band. CPD mRNA was also analysed by Northern blots of four human cell lines (Figure 7). Almost undetectable levels were found in HaCat cells (a keratinocyte cell line), whereas low to moderate levels were seen in Mel Im melanoma cells and HT-29 colon carcinoma cells. High levels of expression were seen in Hep G2 cells, a hepatoma cell line. This is consistent with high levels of immunoprecipitable CPD activity found in solubilized membrane fractions of Hep G2 cells (G. B. McGwire and R. A. Skidgel, unpublished work). It is interesting to note that Hep G2 cells have been reported to synthesize two other members of the regulatory carboxypeptidase family: CPN and CPE [13,14]. The three major bands of 7.4, 6.6 and 5.5 kb found in human tissue RNA were also clearly present in the Hep G2 cells as well as the additional 4.2 kb band found in placenta (Figure 7). The same bands were also evident in the Mel Im RNA, but only the 7.4 kb band was clearly visible in the HaCat cell line (Figure 7). The reason for the multiple forms of CPD mRNA is unknown but they could arise from alternative splicing, the use of multiple transcription start sites or multiple polyadenylation signals. In this regard, it is interesting to note that lower-molecular-mass soluble forms of CPD have been detected [3] that could arise via alternative splicing.

In summary, we have cloned and sequenced the cDNA for human CPD, a membrane-bound B-type carboxypeptidase with an acidic pH optimum, that is highly similar to duck gp180. Duck gp180 is widely distributed in duck tissues and is present on the plasma membrane as well as on intracellular membranes [15]. The physiological function of CPD can only be speculated about at present. The enzymic properties of CPD and its relatively wide distribution indicate that it could potentially be involved in

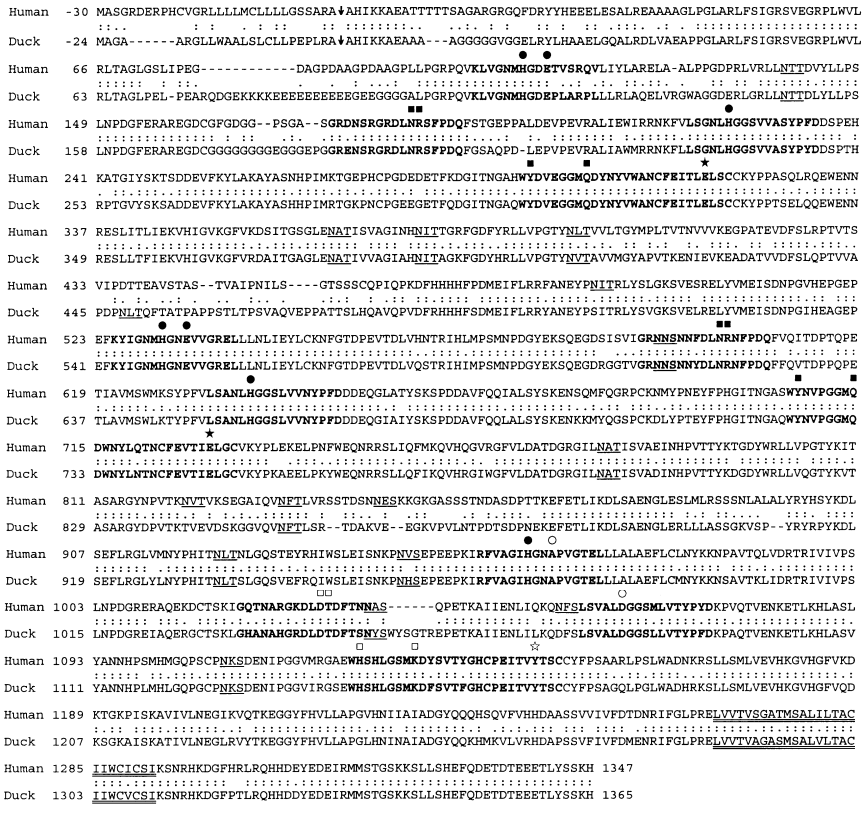

Figure 5 Comparison of the deduced amino acid sequences of human CPD and duck gp180

In the aligned sequences, double dots indicate identical residues, single dots conservative changes, and blanks non-conservative changes. Dashes indicate gaps that were introduced to optimize alignment. Putative active site regions (as in Figure 4) are shown in bold type. Putative active site residues conserved in other members of the regulatory carboxypeptidase family are marked with filled symbols (see the legend to Figure 4 for the key to the symbols). In domain 3, residues that align with active site residues but are not conserved are denoted with open symbols. Potential Asn-linked glycosylation sites are underlined. The putative hydrophobic transmembrane spanning sequence is doubly underlined.

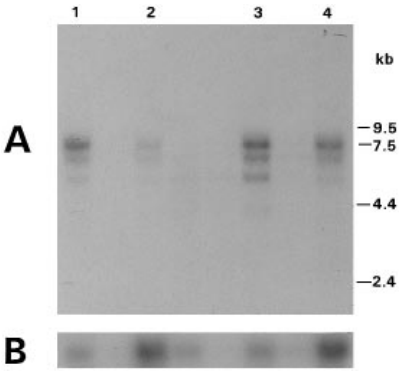

Figure 6 Northern analysis of CPD mRNA in human tissues

A Northern blot of mRNA from human tissues was probed with a CPD-specific probe (**A**) or an oligonucleotide probe specific for 18 S RNA (**B**) as described in the Materials and methods section. Lane 1, pancreas; lane 2, skeletal muscle; lane 3, placenta; lane 4, heart.

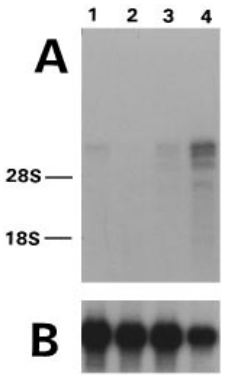

Figure 7 Northern analysis of CPD mRNA in human cell lines

A Northern blot of total RNA from several human cell lines was probed with a CPD-specific probe (**A**) or an oligonucleotide probe specific for 18 S RNA (**B**) as described in the Materials and methods section. Lane 1, HT-29 colon carcinoma cells; lane 2, HaCat keratinocytes; lane 3, Mel Im melanoma cells; lane 4, Hep G2 hepatoma cells.

prohormone/proprotein processing in the constitutive secretory pathway. In addition it could potentially function in endosomes to cleave peptide hormones with C-terminal basic residues (e.g. bradykinin and epidermal growth factor) after receptor-mediated endocytosis.

We thank Dr. Sadoutounnissa Shanmugam for her contribution in obtaining the 2.5 kb PCR clone of human CPD, and Anne Pietryga-Krieger for performing the automated cDNA sequencing. These studies were supported by NIH grants DK41431 and HL36473.

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Received 21 February 1997/28 April 1997; accepted 29 May 1997