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Review

Carboxypeptidases from A to Z: implications in embryonic development and Wnt binding

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Abstract. Carboxypeptidases perform many diverse functions in the body. The well-studied pancreatic enzymes (carboxypeptidases A1, A2 and B) are involved in the digestion of food, whereas a related enzyme (mast-cell carboxypeptidase A) functions in the degradation of other proteins. Several members of the metallocarboxypeptidase gene family (carboxypeptidases D, E, M and N) are more selective enzymes and are thought to play a role in the processing of intercellular peptide messengers. Three other members of the metallocarboxypeptidase gene family do not appear to encode active enzymes; these members have been designated CPX-1, CPX-2 and AEBP1/ACLP. In this review, we focus on the recently discovered carboxypeptidase Z (CPZ). This enzyme removes C-terminal Arg residues from synthetic substrates, as do many of the other members of the gene family. However, CPZ differs from the other enzymes in that CPZ is enriched in the extracellular matrix and is broadly distributed during early embryogenesis. In addition to containing a metallocarboxypeptidase domain, CPZ also contains a Cys-rich domain that has homology to Wnt-binding proteins; Wnts are important signaling molecules during development. Although the exact function of CPZ is not yet known, it is likely that this protein plays a role in development by one of several possible mechanisms.

Key words. Metallocarboxypeptidase; carboxypeptidase Z; extracellular matrix; Frizzle-related proteins; Frizzled receptor; Wnt.

General roles of metallocarboxypeptidases

Carboxypeptidases (CPs) remove amino acids from the C-termini of proteins and peptides by hydrolysis. The cleavage mechanisms use an active site serine, cysteine or zinc; the latter group is referred to as 'metallocarboxypeptidases.' Altogether, there are 13 known members of the metallocarboxypeptidase gene family in most mammalian species investigated; an additional carboxypeptidase A-like member (designated CPA3) has been reported in humans [1]. Several additional CP-like genes are present in the human genome, but it is not yet known whether they encode proteins or are pseudogenes. All metallocarboxypeptidases can be grouped into one of two major subfamilies based primarily on amino acid sequence similarities (fig. 1). One group includes the digestive enzymes carboxypeptidase A (CPA) and carboxypeptidase B (CPB). All members of the CPA/B subfamily are approximately 34–36-kDa proteins initially produced as inactive zymogens [2–6]. Activation requires removal of a pro peptide segment, in some cases by multiple endopeptidase cleavages [7]. The members of this subfamily that have been characterized are optimally active in the neutral pH range, reflecting the pH of the en-

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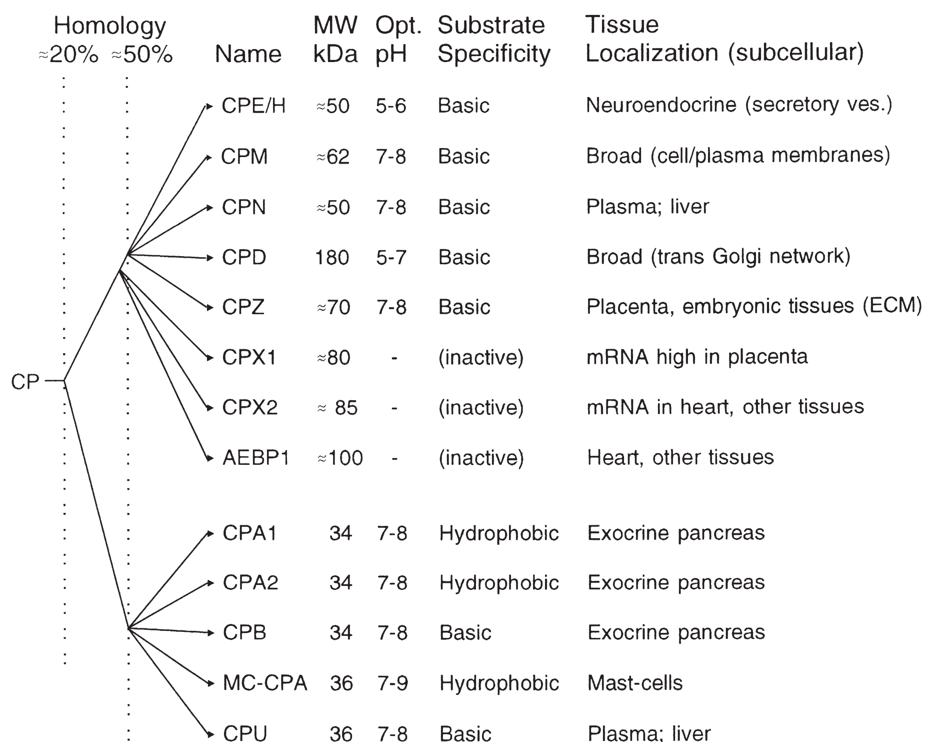

Figure 1. Mammalian metallocarboxypeptidase family tree. The amino acid sequence identity is approximately 50% among members of the same subfamily, but only 20% between subfamilies. Only the members of the metallocarboxypeptidase family that have been characterized at the protein level are included; CPA3 and other possible members are not shown.

vironments where they function [2–6]. The enzymatic properties of CPA3 have not been reported.

In contrast to the members of the CPA/B subfamily, the members of the other metallocarboxypeptidase subfamily range in size and pH optima [8–11]. This second family has been commonly referred to as 'regulatory' carboxypeptidases. However, this is not accurate, because at least one of the members of the A/B subfamily has a regulatory role in fibrinolysis (discussed below). Instead, it is more appropriate to refer to this family as the N/E family, based on the first two members that were identified. Unlike A/B carboxypeptidases, the N/E enzymes do not appear to be produced as inactive precursors that require proteolysis to produce the active form. Instead, the N/E enzymes rely on their substrate specificity and subcellular compartmentalization to prevent inappropriate cleavages that would otherwise damage the cell. In addition, all members of the N/E subfamily contain an extra domain that is not present in the A/B subfamily proteins (fig. 2). Based on the crystal structure of a portion of carboxypeptidase D (CPD) [12] and the modeling of other members of the family [13], this 80 amino acid-long domain folds into a structure that resembles the β barrel found in transthyretin and other proteins. The function of this domain within all members of the N/E subfamily is not known; one possibility is that the transthyretin-like domain functions in the folding of the carboxypeptidase domain. Alternatively, the transthyretin-like domain may be involved in the formation of protein oligomers or regulation of the enzyme activity.

Members of the CPA/B subfamily

The primary function of pancreatic CPA and B is to break down peptides in the gut, following the action of chymotrypsin and trypsin on ingested proteins. Although pancreatic CPA is optimally active towards aromatic or aliphatic residues and pancreatic CPB is optimally active towards basic residues, CPB is able to cleave some nonbasic amino acids [6, 14]. In most mammalian species two distinct pancreatic CPA enzymes are present, named CPA1 and CPA2. These two enzymes have slightly different specificities towards aromatic residues, with CPA2 preferring the bulkier Trp side chain and CPA1 preferring the smaller aromatic and aliphatic residues [2, 3, 15]. In addition to the pancreatic CPAs, a distinct enzyme found in mast cells has also been named CPA and is usually designated mast cell-CPA (MC-CPA). Similar to the pan-

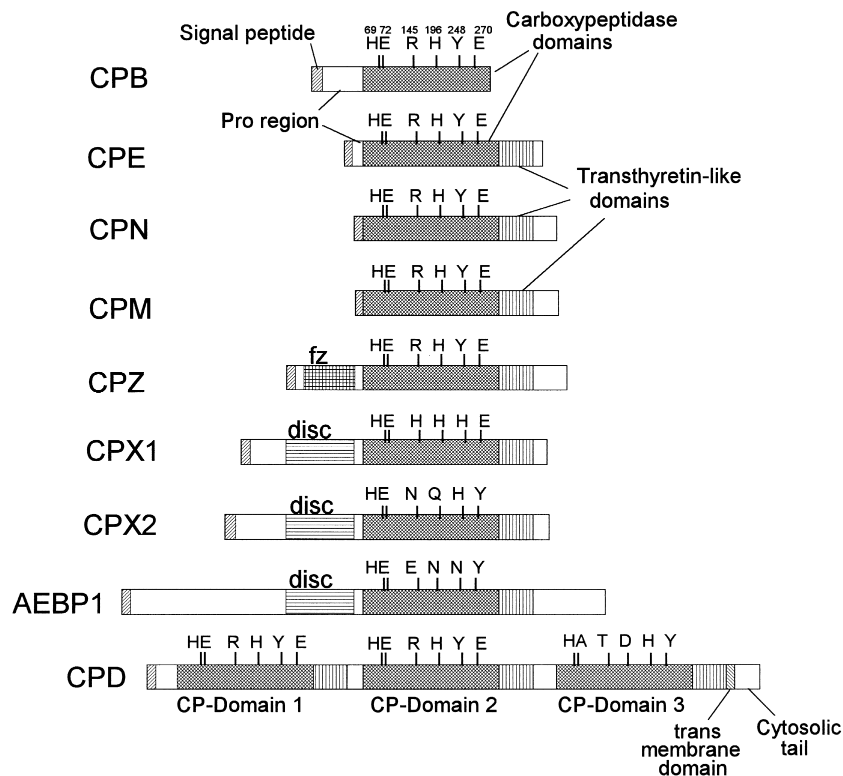

Figure 2. Comparison of the domain structures of metallocarboxypeptidases, with a focus on CPE subfamily members. All members contain an N-terminal signal peptide, a 300-amino acid carboxypeptidase-homology domain, an additional 80-residue domain with structural homology to transthyretin, and then additional sequences at the N- and/or C-termini. Residues that are important for the metal binding (His69, Glu72, His196), substrate binding (Arg145, Tyr248) and catalytic activity (Glu270) are indicated, using the numbering system of CPA/B by convention. Abbreviations: fz, Cys-rich domain with amino acid sequence similarity with Frizzled receptors and other proteins; disc, domain with amino acid similarity to discoidin-1.

creatic CPAs, MC-CPA also cleaves peptides with C-terminal aliphatic/aromatic residues with the highest efficiency [5]. MC-CPA functions in the destruction of proteins and peptides by mast cells, presumably following the action of chymase [5]. Carboxypeptidase U (CPU) is a plasma enzyme that is produced in the liver [4]. This enzyme has also been given the names CPR, plasma CPB, thrombin activatable fibrinolysis inhibitor (TAFI) and inducible carboxypeptidase activity [4]. CPU circulates in plasma primarily in its inactive precursor form, proCPU, which can be activated by either plasmin or thrombin. Once active, CPU cleaves C-terminal lysines formed by the action of plasmin on fibrin. These C-terminal lysines bind plasminogen with high affinity, so the removal of these residues by CPU acts to control the binding of plasminogen which in turn affects the rate of fibrinolysis [4].

CPE

Carboxypeptidase E (CPE), which is also known as carboxypeptidase H, enkephalin convertase, and EC 3.4.17.10 [16], was discovered during searches for a carboxypeptidase that would remove C-terminal lysine and arginine residues from neuroendocrine peptide precursors [17]. Most neuroendocrine peptides are initially produced as small proteins that require cleavage at basic amino acid-containing sites by a prohormone convertase [18]. Following the action of the prohormone convertase, the carboxypeptidase step is then usually required to generate the bioactive peptide. This processing step takes place within the late secretory pathway of neuroendocrine cells where the pH is 5–6, and so the pH optimum of CPE is also within this range [8]. CPE has very little enzyme activity at neutral pH [19], which is presumably a mechanism by which the enzyme is inactivated in the early secretory pathway (with its more neutral pH) and following secretion of CPE into plasma or the synaptic cleft (which are also neutral). CPE is extremely specific for C-terminal basic residues, with no detectable activity towards nonbasic residues [8]. Aside from this stringent requirement for C-terminal basic residues, CPE displays a fairly broad substrate specificity and can efficiently cleave all known peptide-processing intermedi-

ates with the exception of peptides with a proline in the penultimate position [8, 20]. CPE is also broadly distributed in the neuroendocrine system but is not abundant in nonneuroendocrine tissues [21, 22]. Within cells, CPE is further localized to the regulated secretory pathway where it exists in a soluble and a membrane-associated form [23]. These forms arise via differential posttranslational processing of a C-terminal region that presumably forms an amphipathic helix [24]. Although CPE is initially produced with a 14-residue N-terminal extension that is cleaved in a post-Golgi compartment, this pro form is fully active [25]. Based on the broad neuroendocrine distribution and substrate specificity of CPE, it was proposed that this enzyme functions in the biosynthesis of most neuroendocrine peptides [16]. This hypothesis was confirmed by studies on mice lacking CPE activity due to a point mutation in the coding region of the gene [26]. This naturally-occurring '*fat*' mutation replaces the serine in position 202 with a proline, eliminating enzyme activity [26]. Mice homozygous for the *fat* mutation have reduced levels of correctly processed neuroendocrine peptides and greatly elevated levels of the precursors containing C-terminal basic residues [26–30]. It is this decrease in peptide processing that presumably causes the mice to be overweight, although the precise defect has not yet been identified. The general effect of the *fat* mutation on many peptides is consistent with the broad role for CPE in peptide processing. However, these mice do produce a small amount of the correctly processed peptides, implying that an alternative enzyme functions in this pathway. Of all the other known carboxypeptidases, only CPD has the correct specificity and tissue distribution to play a major role in the intracellular biosynthesis of neuroendocrine peptides (discussed below).

CPM and CPN

Carboxypeptidase M (CPM) and carboxypeptidase N (CPN) have also been proposed to function in the processing of peptide hormones, but in contrast to CPE, these other carboxypeptidases function extracellularly. CPM is most abundant in lung and placenta where it is found in a membrane-bound glycosyl-phosphatidylinositol-linked form [11]. It is also present in blood vessels and on the surface of white blood cells where it was discovered as a 'differentiation-dependent cell surface antigen' [11]. In addition, soluble forms of CPM are found in various fluids such as seminal plasma, amniotic fluid and urine [11]. CPN is produced by the liver and circulates in plasma only as a 280-kDa protein complex containing two copies of the active subunit and two copies of an 83-kDa glycoprotein [10]. The major function of both CPM and N may be to inactivate or alter the specificity of vasoactive peptides (kinins and anaphylatoxins C3a, C4a and C5a). In addition, these enzymes may also process peptide hormones that are not fully processed by CPE within the secretory pathway, or regulate plasminogen binding to cells as does CPU (except that CPM and N are constitutively active unlike the thrombin-activated CPU discussed above).

CPD

CPD was first discovered as a 180-kDa duck protein that bound hepatitis B viral particles [31]. Mouse CPD was independently found in a search for a CPE-like enzyme that was functional in mice with the *fat* mutation, and the bovine and rat homologs were subsequently identified [32]. The *Drosophila* homolog of CPD was found to be encoded by the *Silver* gene, mutation of which causes changes in the coloration of the cuticle and in the wing shape [33]. Comparison of the CPD amino acid sequences from human [34], rat [35], mouse [36], duck [31] and *Drosophila* [33] reveals a similar structure; all species contain three repeats of a CPE-like sequence which includes both the 320-residue CPA/B-like domain and then the 80-residue transthyretin-like domain unique to the CPE subfamily proteins (fig. 2). Following these three repeats is a 20-residue transmembrane domain and then a 60-residue cytosolic tail. This transmembrane domain and tail are found in mammalian CPD, avian CPD, and even the *Drosophila* CPD homolog. Although the initial report on the *Silver* gene indicated only two and a half domains with no transmembrane region [33], analysis of the *Drosophila* genome and expressed sequence tag complementary DNA (cDNA) databases reveals that this was due to a splice variant; the unspliced form would contain a transmembrane-domain and cytosolic tail. Similar analysis of the *Caenorhabditis elegans* database indicates the presence of a two-domain CPD homolog, also containing a transmembrane domain and a 60-residue cytosolic tail. CPD from *Aplysia* has four CPE-like repeats but was not reported to contain a transmembrane domain [37]. Thus, the multidomain requirement of CPD has been highly conserved through evolution, although the advantage for these multiple domains is not entirely clear. In the duck and mammalian forms, and presumably the other species as well, the third domain does not have detectable carboxypeptidase activity towards standard substrates [38, 39]. Analysis of the amino acid sequences and modeling of the active site indicates that this domain is unlikely to form a functional carboxypeptidase [13]. It is possible that the third domain represents a distinct hydrolase activity, as found for the distantly related gamma-D-glutamyl-(L)-diamino acid-hydrolyzing peptidase I of *Bacillus sphaericus*. This *B. sphaericus* peptidase is a member of the metallocarboxypeptidase superfamily but lacks some of the residues required for substrate binding

[40]. Similarly, the third domain of CPD lacks some of these same residues. However, the third domain of CPD also lacks several residues that are required for hydrolase activity in both the carboxypeptidases and in the *B. sphaericus* peptidase, so it is also possible that this third domain functions as a binding protein rather than as an active enzyme. Similar enzyme-related binding proteins have been described for a range of proteases and phosphatases [41].

The first and second CPE-like domains of CPD are functional carboxypeptidases and differ slightly in their enzymatic properties [38, 39]. The first domain is optimally active at neutral pH and cleaves peptides with C-terminal arginines more efficiently than peptides with C-terminal lysines. The second domain is optimally active at a more acidic pH range (5–6) and cleaves peptides with C-terminal lysines more efficiently than peptides with C-terminal arginines. Thus, the two domains are somewhat complementary in their activities and provide for the broadest possible specificity over the entire physiological pH range encountered by the enzyme. CPD is enriched in the *trans* Golgi network and also cycles to the cell surface via immature secretory vesicles [42, 43]. The intravesicular pH of these compartments ranges from neutral to acidic and corresponds to the broad pH optimum of CPD. The function of CPD appears to overlap somewhat with that of CPE, which accounts for the viability of mice lacking CPE activity due to the *fat* mutation. However, CPD and E are not entirely redundant, because peptide processing is altered in these mice. Although both CPD and E are present in the secretory pathway of neuroendocrine cells, they are enriched in different parts of the pathway [43]. Similarly, there are also two different sets of endopeptidases within the secretory pathway; one set is enriched in the *trans* Golgi network (furin, prohormone convertase 7 and others), whereas the other set is enriched in the mature secretory vesicles (prohormone convertases 1 and 2) [18]. Thus, CPD primarily functions following the action of the *trans*-Golgi network endopeptidases, whereas CPE functions following the action of prohormone convertase 1 and 2. CPD is therefore likely to be involved in the production of receptors and growth factors which are processed by furin and related enzymes within the *trans*-Golgi network [44]. The tissue distribution of CPD is very broad and is not limited to neuroendocrine tissues, consistent with a broad role for CPD in processing proteins that transit the secretory pathway [18].

CPX1, CPX2 and AEBP1/ACLP

The three proteins designated CPX1, CPX2 and AEBP1 (which is also known as aortic carboxypeptidase-like protein, or ACLP) form a distinct subset of the CPE metallocarboxypeptidase subfamily. First, their carboxypeptidase domains share slightly more amino acid sequence homology with each other than with other members of the metallocarboxypeptidase family (fig. 1). Also, CPX1 and CPX2 are inactive towards standard carboxypeptidase substrates [45, 46], as is the third domain of CPD (discussed above). Although AEBP1/ACLP was reported to have catalytic activity towards a standard CPB substrate [47, 48], these studies could not be replicated with AEBP1/ACLP prepared using the same procedure and with the same substrate [L. D. Fricker, unpublished] or with other expression systems and substrates [49]. CPX1, CPX2 and AEBP1/ACLP all contain an N-terminal domain with homology to discoidin and other lectins, whereas none of the other members of the metallocarboxypeptidase family contain this domain (fig. 2).

A comparison of the active site residues argues against a standard carboxypeptidase function for CPX1, CPX2 and AEBP1/ACLP. All three of these proteins lack one or more residues that are critical for the enzymatic activity and/or substrate binding of the carboxypeptidases. For example, the residue in a position equivalent to Arg145 of CPB is either a His, Asn or Glu in the three inactive proteins (fig. 2). This Arg145 is involved in the binding of the carboxylate group of the substrate and is essential for defining the enzyme as a carboxypeptidase [50]. Without an Arg in this position, it is unlikely that the proteins will bind C-terminal amino acids. Tyr248 of CPB is also important for substrate binding [51], and the equivalent residue is a His in CPX1 and CPX2 and an Asn in AEBP1/ACLP (fig. 2). Glu270 of CPB is critical for the catalytic mechanism; replacement of the Glu in a comparable position in CPE (Glu300) with a Gln eliminated enzyme activity but not substrate binding [52]. The absence of a Glu in the equivalent positions of CPX2 and AEBP1/ACLP (as well as the third domain of CPD) argues that these proteins may function as binding proteins rather than active hydrolases.

Discovery of CPZ

Computer homology searches of expressed sequence tag databases identified a short cDNA sequence with some similarities to CPE and the other carboxypeptidases. Reverse transcription and polymerase chain reaction were used to isolate the full-length cDNA encoding both human and rat CPZ [49, 53]. Human CPZ was found in several forms which arise from alternative splice site usage of exons. The major form encodes a protein of 641 amino acids. Inspection of the deduced amino acid sequence of CPZ suggested that this protein would encode an active carboxypeptidase, unlike CPX1, CPX2 and AEBP1/ACLP. Expression of the protein in the baculovirus system resulted in an active carboxypeptidase [49]. However, the protein was tightly bound to the cells despite the

absence of any apparent transmembrane domains except for the N-terminal region, which was predicted to form a cleavable signal peptide. Studies with nonpermeable substrates confirmed that the expressed CPZ was present on the cell surface, consistent with the presence of a signal peptide to target the protein into the secretory pathway [49]. Recently, expression of a C-terminally truncated form of CPZ in the AtT-20 mammalian cell line has produced a form that is soluble and secreted into the media [54]. The addition of the His6 sequence on the C-terminus of the construct has enabled the purification and subsequent characterization of the enzyme.

CPZ is unique among members of the metallocarboxypeptidase gene family in the presence of a 120-residue cysteine-rich domain that has 20–35% amino acid sequence identity to *Drosophila* and mammalian Frizzled proteins [49, 53]. These proteins are receptors for members of the Wingless/Wnt family, which are important signaling molecules in early development (discussed below). Several extracellular proteins also contain this Frizzled-like cysteine-rich domain, including a form of collagen, proteins designated frzb and sizzled, and a family of Frizzled-related proteins [55–58] (fig. 3). These proteins are believed to bind to Wnt proteins extracellularly, thus inhibiting Wnts from interacting with Frizzled receptor proteins and influencing gene expression. The cysteine-rich domain within CPZ is highly conserved between the human and rat forms of the protein, suggesting that this domain is functional. Since CPZ is not a transmembrane-spanning protein, it more likely functions as an extracellular Wnt-binding protein than as a receptor. Recently, we have shown that Wnt-3a induced transformation of the mouse mammary gland cell line C57MG cells could be antagonized by CPZ [S. Reznik, unpublished].

Localization of CPZ in the extracellular matrix

To investigate whether CPZ is present in the parts of cells where an interaction with Wnt proteins is possible, the intracellular distribution of endogenous CPZ was examined in several human tissues and in two cell lines; the rat adrenal PC12 cell line and the mouse pituitary AtT20 cell line. PC12 cells express relatively high levels of CPZ. AtT-20 cells do not express detectable levels of CPZ, and so these cells were transfected with human CPZ cDNA. CPZ was found in the extracellular matrix fraction of both the PC12 and AtT-20 cells [54]. In addition, CPZ was detected in the extracellular space of human placental invasive trophoblasts and in the extracellular matrix adjacent to malignant cells in human adenocarcinoma of the colon (fig. 4).

Similar to other components of the extracellular matrix, CPZ binds heparin [54]. The addition of heparin to the cell culture medium does not significantly alter the amount of CPZ secreted into the media or bound to the extracellular matrix, indicating that the binding of CPZ to the extracellular matrix is not simply due to the heparin-binding domain of CPZ [54]. It is likely that the C-terminal region of CPZ contributes to the extracellular matrix binding for the following reasons. The form of CPZ expressed in the media of CPZ-expressing AtT-20 cells is approximately 4 kDa smaller than the extracellular matrix form in these cells [54]. Thus, it is likely that the extracellular matrix binding is contained within this additional 4-kDa portion of the larger form. This 4-kDa difference is not due to N-linked carbohydrates, and so the likely explanation is proteolysis at the N- and/or C-termini. A comparison of rat and human CPZ sequences shows a highly conserved C-terminal stretch of 35 amino acids [49, 53]. The degree of amino acid identity over these C-terminal 35 residues (78%) is considerably higher than that found in an adjacent region (36%), and it is likely that such a highly conserved region is functional. Furthermore, there are eight basic residues and no acidic residues in the C-terminal domain, and these eight basic residues are conserved in human and rat [49, 53]. A consensus site for furin and related prohormone convertases is located 29 residues from the C-terminus. Cleavage at this consensus site by furin would shorten CPZ by 3.6 kDa, consistent with the observed shift of approximately 4 kDa. Although the majority of intracellular furin is found in the *trans*-Golgi network, this endopeptidase also cycles to the cell surface and has been shown to process several extracellular proteins [44, 59]. Thus, furin could process CPZ during transport to the surface of the cell, or following expression of CPZ on the surface of the cell.

CPZ Expression in embryonic development

The presence of the N-terminal Frizzled domain within CPZ and the localization of CPZ in the extracellular matrix suggest that CPZ interacts with Wnt proteins. Because Wnt proteins function in the regulation of embryonic development, it is possible that CPZ is also involved. CPZ expression was detected as early as E5 in the mouse embryo, as well as in the mouse decidualized endometrium [60]. CPZ immunoreactivity intensifies in the embryonic bilayer at E7 and is broadly expressed in the embryo at E9 and E12, when immunostaining is most intense (fig. 5, panels A, B). In E12 brain, staining is most abundant in the cells lining the ventricles (fig. 5, panel E). At E15 there is a decrease in CPZ expression in specific tissues. In particular, there is a dramatic fall in CPZ immunoreactivity from brain parenchyma, but ventricular and cochlear lining cells continue to show strong CPZ expression (fig. 5, panel F). Intense CPZ immunoreactivity also persists in the lungs, liver, kidneys and intestine at

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               *         *          *    *                         *         *    * *           *   *      *          **    *             *
Human CPZ     TCVDLQ--LRTCSDAAYNHTTFPNLLQHRSWEVVEASSEYILLSVLHQLLEGQCNPD-LRLLGCAVLAPRCEGGWVR---RPCRHICEGLREVCQPAFDAIDMAWPYFLDCHRYFTRE---DEGCY
Rat CPZ       TCVDLH--LRTCADAAYNHTSFPTPLEHRSWEAVEASPEYTLLGVLHFLLEGQCNPD-LRLLGCSVLAPRCQGGHTQ---RPCRRVCEGLREACQPAFDAIDMAWPYFLDCTQYFAPE---EEGCY
Human frz1    YCQPIS--IPLCTDIAYNQTIMPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSAE-LKFFLCSMYAPVCTVLEQA--LPPCRSLCERARQGCEALMNKFGFQWPDTLKCEKFPVHG--AGELCV
Rat frz1      YCQPIS--IPLCTDIAYNQTIMPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSAE-LKFFLCSMYAPVCTVLEQA--LPPCRSLCERA-QGCEALMNKFGFQWPDTLKCEKFPVHG--AGELCV
Mouse frz1    YCQPIS--IPLCTDIAYNQTIMPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSAE-LKFFLCSMYAPVCTVLEQA--LPPCRSLCERARQGCEALMNKFGFQWPDTLKCEKFPVHG--AGELCV
C.eleg frz1   RCQKVD--HEMCNDLPYNLTSFPNLVDEESWK--DASES---ILTYKPLLSVVCSEQ-LKFFLCSVYFPMCNEKLAN-PIGPCRPLCLSVQEKCLPVLESFGFKWPDVIRCDKFPLENN-REKMCM
Dros. frz     RCEPIT--ISICKNIPYNMTIMPNLIGHTKQE--EAGLE---VHQFAPLVKIGCSDD-LQLFLCSLYVPVCTILERP--IPPCRSLCESA-RVCEKLMKTYNFNWPENLECSKFPVHG--GEDLCV
Human frz2    FCQPIS--IPLCTDIAYNQTIMPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSPE-LRFFLCSMYAPVCT-VLEQ-AIPPCRSICERARQGCEALMNKFGFQWPERLRCEHFPRHG--AEQICV
Rat   frz2    FCQPIS--IPLCTDIAYNQTIMPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSPE-LRFFLCSMYAPVCTVLEQA--IPPCRSICERARQGCEALMNKFGFQWPERLRCEHFPRHG--AEQICV
Dros. frz2    RCEEIT--IPMCRGIGYNMTSFPNEMNHETQD--EAGLE---VHQFWPLVEIKCSPD-LKFFLCSMYTPICLEDYHK-PLPVCRSVCERARSGCAPIMQQYSFEWPERMACEHLPLHGD-PDNLCM
Human frz3    SCEPIT--LRMCQDLPYNTTFMPNLLNHYDQQ--TAALA---MEPFHPMVNLDCSRD-FRPFLCALYAPICMEYGRV--TLPCRRLCQRAYSECSKLMEMFGVPWPEDMECSRFP-------DCD
Mouse frz3    SCEPIT--LRMCQDLPYNTTFMPNLLNHYDQQ--TAALA---MEPFHPMVNLDCSRD-FRPFLCALYAPICMEYGR--VTLPCRRLCQRAYSECSKLMEMFGVPWPEDMECSRFP-------DCD
Human frz4    RCDPIR--ISMCQNLGYNVTKMPNLVGHELQT--DAELQ---LTTFTPLIQYGCSSQ-LQFFLCSVYVPMCTEKINI-PIGPCGGMCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQND-HNHMCM
Mouse frz4    RCDPIR--IAMCQNLGYNVTKMPNLVGHELQT--DAELQ---LTTFTPLIQYGCSSQ-LQFFLCSVYVPMCTEKINI-PIGPCGGMCLSVKRRCEPVLREFGFAWPDTLNCSKFPPQND-HNHMCM
Human frz5    VCQEIT--VPMCRGIGYNLTHMPNQFNHDTQD--EAGLE---VHQFWPLVEIQCSPD-LRFFLCTMYTPICLPDYHK-PLPPCRSVCERAKAGCSPLMRQYGFAWPERMSCDRLPVLGRDAEVLCM
Human frz6    TCEPIT--VPRCMKMAYNMTFFPNLMGHYDQS--IAAVE---MEHFLPLANLECSPN-IETFLCKAFVPTCIEQIHV--VPPCRKLCEKVYSDCKKLIDTFGIRWPEELECDRLQ-------YCD
Mouse frz6    TCEPIT--VPRCMKMTYNMTFFPNLMGHYDQG--IAAVE---MGHFLHLANLECSPN-IEMFLCQAFIPTCTEQIHV--VLPCRKLCEKIVSDCKKLMDTFGIRWPEELECNRLP-------HCD
Human frz7    FCQPIS--IPLCTDIAYNQTILPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSPE-LRFFLCSMYAPVCTVLDQA--IPPCRSLCERARQGCEALMNKFGFQWPERLRCENFPVHG--AGEICV
Mouse frz7    FCQPIS--IPLCTDIAYNQTILPNLLGHTNQE--DAGLE---VHQFYPLVKVQCSPE-LRFFLCSMYAPVCTVLDQA--IPPCRSLCERARQGCEALMNKFGFQWPERLRCENFPVHG--AGEICV
Human frz8    ACQEIT--VPLCKGIGYNYTYMPNQFNHDTQD--EAGLE---VHQFWPLVEIQCSPD-LKFFLCSMYTPICLEDYKK-PLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGN-PDTLCM
Mouse frz8    ACQEIT--VPLCKGIGYNYTYMPNQFNHDTQD--EAGLE---VHQFWPLVEIQCSPD-LKFFLCSMYTPICLEDYKK-PLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGN-PDTLCM
Human frz9    PCQAVE--IPMCRGIGYNLTRMPNLLGHTSQG--EAAAE---LAEFAPLVQYGCHSH-LRFFLCSLYAPMCTDQVST-PIPACRPMCEQARLRCAPIMEQFNFGWPDSLDCARLPTRND-PHALCM
Mouse frz9    PCQAME--IPMCRGIGYNLTRMPNLLGHTSQG--EAAAQ---LAEFSPLVQYGCHSH-LRFFLCSLYAPMCTDQVST-PIPACRPMCEQARLRCAPIMEQFNFGWPDSLDCARLPTRND-PHALCM
Human frz10   KCQPIE--IPMCKDIGYNMTRMPNLMGHENQR--EAAIQ---LHEFAPLVEYGCHGH-LRFFLCSLYAPMCTEQVST-PIPACRVMCEQARLKCSPIMEQFNFKWPDSLDCRKLPNKND-PNYLCM
MouseSFRP1    QCVDIPVDLRLCHNVGYKKMVLPNLLEHETMA--EVKQQ---ASSWVPLLNKNCHMG-TQVFLCSLFAPVC----LDRPIYPCRWLCEAVRDSCEPVMQFFGFYWPEMLKCDKFPEG-----DVCI
MouseSFRP2    NCKPIPANLQLCHGIEYQNMRLPNLLGHETMK--EVLEQ---AGAWIPLVMKQCHPD-TKKFLCSLFAPVCLDD-LDETIQPCHSLCVQVKDRCAPVMSAFGFPWPDMLECDRFPQDN----DLCI
Human frzb    ACEPVR--IPLCKSLPWNMTKMPNHLHHSTQA--NAILA---IEQFEGLLGTHCSPD-LLFFLCAMYAPICTIDFQHEPIKPCKSVCERARQGCEPILIKYRHSWPENLACEELPVYD---RGVCI
Mouse frzb    ACEPVR--IPLCKSLPWNMTKMPNHLHHSTQA--NAILA---MEQFEGLLGTHCSPD-LLFFLCAMYAPICTIDFQHEPIKPCKSVCERARQGCEPILIKYRHSWPESLACDELPVYD---RGVCI
Xen. frzb     SCEPVR--IPMCKSMPWNMTKMPNHLHHSTQA--NAILA---IEQFEGLLTTECSQD-LLFFLCAMYAPICTIDFQHEPIKPCKSVCERARAGCEPILIKYRHTWPESLACEELPVYD---RGVCI
Hum. SFRP4    PCEAVR--IPMCRHMPWNITRMPNHLHHSTQE--NAILA---IEQYEELVDVNCSAV-LRFFFCAMYAPICTLEFLHDPIKPCKSVCQRARDDCEPLMKMYNHSWPESLACDELPVYD---RGVCI
Hum. SFRP5    QCLDIPADLPLCHTVGYKRMRLPNLLEHESLA--EVKQQ---ASSWLPLLAKRCHSD-TQVFLCSLFAPVC----LDRPIYPCRSLCEAVRAGCAPLMEAYGFPWPEMLHCHKFPL-D---NDLCI
Xen. sizz     KCVTIPTEMAMCNDVGYSEMRLPNLMGHTNMA--EVVPK---SAEWQNLLQTGCHPYAARTFLCSLFAPVCLDTF----IQPCRSMCVAVRDSCAPVLACHGHSWPESLDCDRFPAG----EDMCL
Mouse coll    RCLPLPPTLTLCSRLGIGHFWLPNHLHHTDSVEVEATVQ-----AWGRFLHTNCHPF-LAWFFCLLLAPSCGPGPPP-PLPPCRQFCEALEDECWNYLAGDRL--P--VVCASLPSQE---DGYCV
consensus      C  h   h hC  h YN T hPN h H       A      h  h  hh   C    h hFLC hh P C          PC+ hC-     C  hh  h   WP- h C  hP         C
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Figure 3. Comparison of the Cys-rich domain within CPZ with a region of Frizzled receptors and other Wnt-binding proteins. * (top line) indicate residues conserved in all, or nearly all of the sequences. A consensus site is indicated (bottom line): h, hydrophobic residues; +, basic residues (Lys, Arg, His); –, acidic residues (Asp, Glu). Other conserved residues are indicated using the single-letter amino acid code. The alignment of some of the sequences was based in part on alignments indicated in the Web site http://www.stanford.edu/~rnusse/wntwindow.html. Abbreviations: C. eleg, *Caenorhabditis elegans*; Dros., *Drosophila melanogaster*; Xen., *Xenopus laevis*; Hum., human; sizz, sizzled; coll, type XVIII collagen.

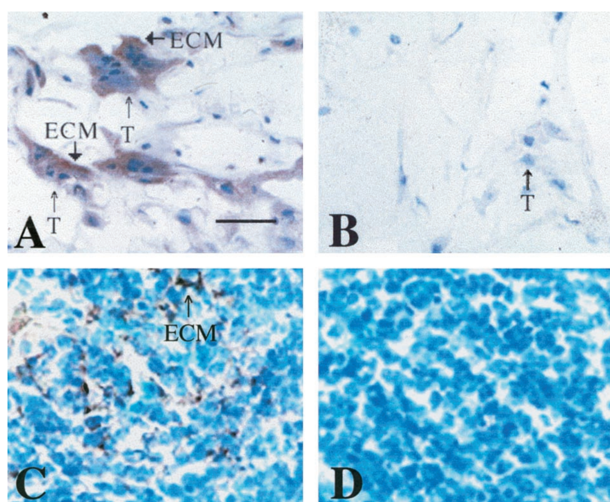

Figure 4. CPZ in the extracellular matrix. Frozen tissue sections were fixed in paraformaldehyde and reacted with antiserum directed against the C-terminal region of CPZ (*A* and *C*) or preimmune serum (*B* and *D*), as previously described [54]. Immunoreactivity was visualized with an avidin-biotin complex developer kit and 3,3′-diaminobenzidine as substrate. *A* and *B*, placental basal plate. *C* and *D*, adenocarcinoma of the colon. Abbreviations: T, invasive trophoblast; ECM, extracellular matrix. Bar, 10 μm.

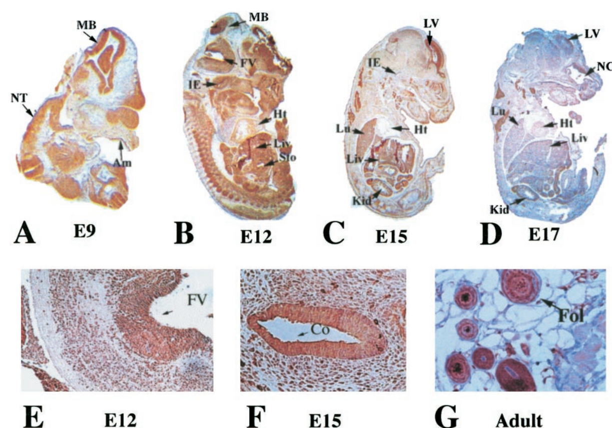

Figure 5. CPZ expression in mouse tissues. An overview of CPZ expression in sagittal sections of mouse embryos is shown in panels *A–D*. Peak CPZ expression is present at E9 and E12 (*A*, *B*). CPZ immunoreactivity decreases in some tissues at E15 (*C*) and falls further at E17 (*D*). Intense CPZ immunoreactivity is present throughout gestation in the brain ventricular epithelial cells (cells lining fourth ventricle at E12 shown in *E*). CPZ expression also persists throughout gestation in the cochlear lining cells of the inner ear and surrounding mesenchymal cells (*F*). CPZ immunoreactivity is present in hair follicles in adult mouse skin (*G*).

E15 (fig. 5, panel C). By E17 CPZ immunoreactivity is markedly decreased in most mouse tissues (fig. 5, panel D). CPZ expression persists throughout development in inner ear cochlear epithelial cells and surrounding mesenchyme, ventricular lining cells in the brain and cartilaginous condensations and surrounding connective tissue in ribs. Mouse placental tissues show uniformly high CPZ expression throughout development both in the amnion and in the fetal chorionic villi [60].

Consistent with its role in embryonic development, CPZ expression in adult tissues is much less abundant than in embryonic organs. By Northern blot analysis of rat tissues, CPZ is expressed at low levels in a variety of adult tissues and at high levels in placenta [53]. By immunohistochemical analysis of mouse tissues, CPZ expression is found in surface epithelial cells and hair follicles in adult skin (fig. 5, panel G). Adult mouse brain and liver show very low CPZ expression, in contrast to high levels of CPZ expression seen in these tissues during embryogenesis [60]. Interestingly, in the adult mouse spleen, CPZ expression is enriched in the extracellular matrix [60]. In placenta, CPZ is enriched in the invasive trophoblasts and the amnionic epithelial cells [54].

The expression pattern of CPZ during mouse embryonic development overlaps with the expression patterns of multiple *Wnt* genes. Several *Wnts* are maximally expressed during the first half of mouse development [61–65]. The expression pattern of the *Wnt-5a* gene in particular is similar to the expression pattern of CPZ. *Wnt-5a* gene expression occurs at E6.5 in decidualized endometrium, at E9.5 in the midbrain and at E11.5–E14.5 in cartilaginous condensations and surrounding mesenchyme [65]. Similar to CPZ, *Wnt* genes have very restricted expression in mouse adult organs. For example, the *Wnt-3* gene is expressed in adult mouse skin, but only in specific cells in the hair follicle [66]. The overlap in patterns of expression of CPZ and several *Wnt* genes is consistent with the putative role of CPZ in interacting with Wnt proteins and regulating aspects of embryonic development controlled by Wnt signaling.

Enzymatic properties of CPZ

The enzymatic properties of CPZ have been characterized with protein purified from homogenates of CPZ-expressing AtT-20 cells. The pH optimum of CPZ is 7–8 with two synthetic substrates tested, Dansyl-Phe-Ala-Arg or Dansyl-Pro-Ala Arg [67]. CPZ is approximately 10% as active at pH 5.5 as in the optimal pH range. As expected for a metallocarboxypeptidase, CPZ is strongly inhibited by the chelating agents 1,10-phenanthroline, EDTA or EGTA. Not surprisingly, CPZ activity is unaffected by serine protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine) or by most cysteine protease inhibitors (iodoacetamide), although 0.1 mM concentrations of another cysteine-directed reagent (*p*-chloromercuriphenylsulfonate) inhibits the enzyme. Interestingly, tosyl-lysyl-chloromethyl ketone, a trypsin inhibitor, but not the related tosyl-phenylalanyl-chloromethyl ketone partially inhibits CPZ activity. Guanidinoethylmercaptosuccinic acid, an active site-directed inhibitor of metallo-

carboxypeptidases, including CPE [68–69, 32], inhibits CPZ with an IC_{50} (concentration at which 50% of the enzyme activity is inhibited) of approximately 10 µM. The IC_{50} of the sulfhydryl analog 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid is approximately one order of magnitude higher. Divalent cations at millimolar concentrations either inhibit (Zn^{2+}, Mn^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+}) or have no effect (Ca^{2+}, Mg^{2+}) on the enzyme activity.

The relative affinity of CPZ for C-terminal Lys versus C-terminal Arg was examined by testing the ability of two peptides, hippuryl-Arg and hippuryl-Lys, to compete for CPZ hydrolysis of dansyl-Phe-Ala-Arg. Hippuryl-Arg inhibits hydrolysis of the dansylated tripeptide with an IC_{50} of approximately 10 mM, whereas the same concentration of hippuryl-Lys does not affect CPZ activity. The substrate specificity of CPZ has been further characterized, using a series of dansylated tripeptides. CPZ cleaves dansyl-Phe-Ala-Arg and dansyl-Pro-Ala-Arg most rapidly of the substrates examined, with K_m values for both substrates of approximately 2 mM. Only 5–10% of the dansyl-Phe-Gly-Arg and 3–5% of the dansyl-Phe-Phe-Arg are cleaved under conditions where dansyl-Phe-Ala-Arg and dansyl-Pro-Ala-Arg are 50% hydrolyzed. Dansyl-Phe-Pro-Arg and dansyl-Phe-Ile-Arg are not cleaved by CPZ.

It is unlikely that CPZ plays a broad role in neuroendocrine peptide processing, given its neutral pH optimum, narrow substrate specificity and limited distribution. The pattern of expression of CPZ is entirely different from that of CPE. Although CPZ is expressed in most tissues examined, it is localized to a restricted number of specific cell types within each organ. In adult brain, for example, CPZ is primarily expressed in leptomeningeal cells. CPE, on the other hand, is generally expressed only in neuroendocrine tissues, but in many cell types in those tissues. Few cells, therefore, express both CPE and CPZ, making it unlikely that the two enzymes have overlapping functions in intracellular neuroendocrine peptide processing. It is likely that CPZ performs a catalytic function apart from the processing of intracellular neuroendocrine peptides, perhaps with components of the extracellular matrix as substrates, since, unlike CPE, CPZ is active at the neutral pH of the extracellular environment.

Wnt proteins

As mentioned above, the presence of the Frizzled domain within CPZ, the localization of the protein in the extracellular matrix and the overlap of CPZ expression during embryonic development with several *Wnt* genes suggest that CPZ may function as a Wnt-binding protein. The Wnt family consists of signaling proteins that have homology to Wnt-1 in the mouse (initially named int-1) [70–71] and wingless (Wg) in *Drosophila* [72–73]. Members of the Wnt family are glycoproteins, typically 350–400 amino acids in length, with a minimum degree of sequence identity of 18%, plus at least 23–24 conserved cysteine residues, in addition to other conserved amino acid residues. Homologous genes encoding Wnt proteins occur in organisms as varied as mammals and the nematode *C. elegans*. Vertebrates generally have pairs of very similar *Wnt* genes, referred to as A–B pairs. Work with Wnt proteins has been hampered by the extreme difficulty in obtaining purified protein or antisera that recognize the Wnt proteins. Wnt action can be indirectly monitored, however, by testing for the stabilization of *β*-catenin, a component of the Wnt signal transduction pathway [74]. Alternatively, transforming Wnts can induce morphological transformation of target cells [75].

Much of what is known about the function of Wnt proteins has been learned from studies involving whole organisms. These experiments have revealed the role of Wnt proteins in the regulation of normal embryonic development. For example, disruption of the gene encoding Wnt-1 in mice results in neural defects [76]. Interestingly, ectopic expression of Wnt-1 in embryonic cells leads to several striking phenotypes, including a duplication of the embryonic axis in *Xenopus* leading to the development of two-headed tadpoles, an increase in the number of mitogenic cells in the ventricular region of the mouse spinal cord and stimulation of the metanephric mesenchyme to differentiate into glomerular and renal tubular epithelia in the mouse kidney [77–79]. Disruption of the gene encoding Wnt-7a results in mice with ventralized limbs, consistent with the observation that *Wnt-7a* is expressed in the dorsal epidermis [80]. Furthermore, in gain of function experiments where *Wnt-7a* is ectopically expressed in chicken limb buds, the chick embryo's limbs are dorsalized [81]. Mice with a disruption of the *Wnt-4* gene do not develop kidneys [82]. Consistent with this phenotype, ectopic expression studies of *Wnt-4* show that it may function in the mesenchymal-epithelial transitions occurring during the formation of the kidney [79].

In addition to their role in the regulation of embryonic development, Wnts are implicated in oncogenesis, as deregulated Wnt signaling can cause cellular transformation [83]. Mouse Wnts have been grouped into three categories, depending on the ability of each Wnt to transform sensitive cells. Wnt-1, Wnt-3a and Wnt-7a are highly transforming, Wnt-2, Wnt-5b and Wnt-7b are intermediate in their ability to transform cells and Wnt-4, Wnt-5a and Wnt-6 are considered nontransforming Wnts [84]. Transforming Wnts have so far been implicated in the production of at least four tumor types: adenocarcinoma of the colon, malignant melanoma, hepatocellular carcinoma [85] and mammary tumors [70].

Wnt signal transduction

Wnt proteins serve as ligands for members of the Frizzled family, which are membrane-spanning proteins that function in the transduction of the Wnt signal to the cytoplasm. Signal transduction occurs via three distinct pathways, depending on whether the bound Wnt is transforming or nontransforming. When a transforming Wnt binds to a Frizzled receptor, the cytoplasmic protein known as Disheveled is hyperphosphorylated and activated [86]. The activation of Disheveled leads in turn to the suppression of the activity of the serine/threonine kinase glycogen synthase kinase 3 (GSK 3). Active GSK 3 promotes the degradation of β-catenin, a multifunctional protein also regulated by adenomatous polyposis coli protein and homologous to the *Drosophila* protein known as armadillo. Suppression of GSK 3 by Wnts, therefore, antagonizes β-catenin degradation and leads to increased intracellular concentrations of the armadillo homolog in mammalian cells. β-catenin interacts with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of architectural transcription factors in the nucleus. Suppression of GSK 3 leading to the stabilization of β-catenin, therefore, allows more interactions between β-catenin and LEF/TCF transcription factors. LEF/TCF family members then bind consensus sites in promoters and induce transcription of Wnt responsive genes. In con trast, signal transduction for nontransforming Wnts is independent of the β-catenin-triggered system and is mediated by either the phosphatidylinositol cycle via a G-protein-coupled pathway or by the Jun-kinase pathway [87].

Wnt-binding proteins

Members of the Frizzled family of transmembrane receptors mentioned above contain a C-terminal cytoplasmic tail, seven membrane-spanning sequences and the extracellular cysteine-rich Wnt binding domain. Bhanot et al. [88] were the first to show that Frizzled proteins functioned as receptors for Wnt proteins. *Drosophila* S2 cells transfected with *Drosophila Frizzled-2* responded to the *Drosophila* Wnt protein known as Wg, whereas wild-type S2 cells were unresponsive to Wg. Loss-of-function experiments in *Drosophila* provided more evidence for a role of Frizzled proteins in Wnt signaling. Mutations in the *Drosophila* gene encoding Frizzled-1, the first Frizzled protein discovered, result in a tissue polarity defect. Normally, *Drosophila* wing blade epithelial cells are aligned, allowing the wing hairs, of which there are one per cell, to all point in a distal direction. In flies lacking the *Frizzled* gene, epithelial cells in the wing blade become disrupted, and wing hairs are oriented haphazardly. Disruption of the *Frizzled* gene has a similar effect on the orientation of bristles on the notum and legs [89], and on the orientation of the ommatidia in the insect eye [90].

While at least 18 mammalian *Wnt* genes have been identified, only 11 mammalian Frizzled receptor genes are known, suggesting that some receptors can bind more than one type of Wnt. In addition to Frizzled receptors binding multiple Wnt proteins, it is also likely that Wnt proteins bind multiple Frizzled receptors. In support of this, Wg was found to interact with *Drosophila* Frizzled-1, *Drosophila* Frizzled-2, mouse Frizzled-4, human Frizzled-5, mouse Frizzled-7, and mouse Frizzled-8, but not mouse Frizzled-3.

The cysteine rich Wnt binding domain does not occur only in Frizzled membrane-spanning proteins, but has been found to occur in a family of secreted proteins. These secreted Frizzled-related proteins (sFRPs) have a size of approximately 30 kDa and contain a putative signal sequence, followed by the Frizzled-like domain and then a conserved hydrophilic carboxy-terminal domain. The sFRPs are encoded by distinct genes and are not the products of alternate splicing of the transmembrane Frizzled protein genes. Binding of the sFRPs to Wnt proteins was confirmed by anchoring derivatives of sFRP-2 and sFRP-3 with glycosylphosphatidylinositol, expressing the protein in human embryonic kidney cells, and then observing cell surface binding by Wg. One of the sFRPs was identified during a search for cartilage- and bone-inducing activities in bovine cartilage extracts. cDNAs encoding proteins involved in induction of cartilage and bone and expressed in the human limb bud were found also to contain regions encoding a Frizzled domain. The protein encoded by these genes was named Frzb [91] and also SFRP3. The *Xenopus* homolog of Frzb is expressed in the dorsal marginal zone of the embryo, termed the Spemann organizer, which controls the patterning of the equatorial region of the frog embryo. Another secreted protein containing a Frizzled domain has been named sizzled (secreted Frizzled) and is expressed in a narrow domain in the ventral marginal zone of *Xenopus* embryos. Both Frzb and sizzled have been shown to antagonize *Xenopus* Wnt-8. Ectopic expression of *Xenopus* Wnt-8 in the ventral side of early cleaving frog embryos results in the development of two-headed tadpoles [92]. Interestingly, ectopic expression of the same Wnt protein later in embryonic development causes ventralization of the embryo [93]. When Frzb is coinjected with *Xenopus* Wnt-8, the disruption in the developing embryo is prevented [55, 94]. Similarly, *Xenopus* Wnt-8 induced ventralization of *Xenopus* embryos is also blocked by sizzled [56].

Several genes encoding extracellular proteins containing Frizzled domains have now been identified, including additional members of the Frzb family, one form of collagen VIII and, as we have described above, CPZ [55, 91, 95–97]. Although antagonism of Wnt action has been demonstrated for some of these secreted Frizzled related

proteins, it is not known whether they all function to downregulate Wnt effects, and whether the non-Frizzled domain in these proteins, e.g. the bone-developing domain of Frzb or the catalytic domain of CPZ, also plays a role in modulation of Wnt function.

Models of CPZ function

Although the exact function of CPZ is not yet known, it is likely that this protein plays a role in development by one of several possible mechanisms (fig. 6). First, CPZ may function in the regulation of embryonic development by interacting with Wnt proteins (fig. 6, model 1). The localization of the gene encoding CPZ is consistent with this putative role in development. Using radiation hybrid mapping as previously described [98], we have found the CPZ gene at human chromosome 4p16.3. The gene is located 6.6 cR from the STS D4S394 in the interval between D4S394 and WI-6518 in the H3 isocore family. Interestingly, the gene encoding BAPX1, a homeobox gene expressed in mouse embryonic skeleton and currently a candidate for human disorders of skeletal development [99–100], is located only 2–4 cR (centiRay) proximal to the gene encoding CPZ. At least two additional homeobox genes, H6 and HOX7, also are located on the short arm of human chromosome 4 [101]. At least five human diseases are associated with 4p deletions, and all are characterized by abnormalities of skeletal development: craniosynostosis Adelaide type [102], Ellis-van Creveld syndrome [103], Weyers acrofacial dysostosis [104], achondro/hypochondroplasia [105–106] and Wolf-Hirschhorn/Pitt-Rogers-Danks syndrome [107]. This observation is particularly interesting, because we have shown that CPZ is expressed in mouse embryonic rib chondrocytes [60]. Finally, Trisomy 4p occurs in humans and is characterized by abnormal development of the face, head and extremities [108].

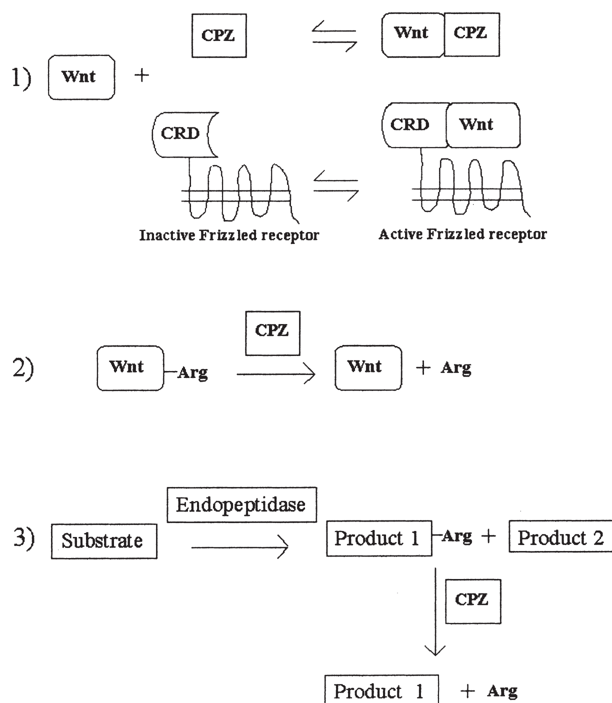

Figure 6. Models of CPZ Function. CPZ may function as a secreted Frizzled-related protein and antagonize Wnt signaling by binding Wnts in the extracellular environment, thus preventing them from binding to the cysteine-rich domain (CRD) of the Frizzled receptors and activating these receptors (model 1). CPZ may remove the C-terminal Lys or Arg residue present on many of the Wnts. Although there is no known function for this modification, it is possible that this activates or inactivates the Wnt, renders it susceptible to further degradation or alters its targeting within the extracellular matrix (model 2). Finally, the role of CPZ may be to enhance the activity of the endoproteases that reside in and act on the extracellular matrix, by removing the C-terminal basic residue from one of the products of an endopeptidase reaction and relieving product inhibition (model 3).

In addition to being encoded by a gene that lies in a chromosomal region associated with embryonic development, CPZ displays a dynamic pattern of expression over the course of development in the mouse [60] (fig. 5), and this pattern overlaps with the pattern of expression of multiple murine *Wnt* genes. Furthermore, CPZ is found in the extracellular space, where the enzyme can access Wnts, which are also secreted into the extracellular environment. Finally, CPZ contains a domain predicted to interact with Wnts by amino acid sequence homology with other Wnt binding proteins. CPZ may function like other secreted Frizzled proteins (members of the Frzb family, sizzled and so on) by competing with Frizzled transmembrane receptors for Wnt binding (fig. 6, model 1). The enzyme may thus antagonize the function of Wnts by preventing the activation of Wnt-induced signal transduction pathways, thereby affecting transcription of specific genes involved in embryonic development.

As certain Wnts have been implicated in oncogenesis, CPZ may function as a tumor suppressor by downregulating Wnt action. We have recently found that CPZ antagonizes transformation of C57MG cells induced by mouse Wnt-3a [unpublished]. Interestingly, the region of human chromosome 4 that contains the CPZ gene also contains one or more genes associated with the suppression of specific cancers. A deletion of the distal 34 cM of 4p, which overlaps with the location of the CPZ gene, leads to the development of neuroblastomas [109]. However, this is a very large deletion that contains many other genes, and it is premature to conclude that CPZ is the tumor suppressor gene in this region.

In addition to CPZ, other proteins (such as frzb) contain a Frizzled-homology domain adjacent to another seemingly unrelated domain. Although not related by amino acid sequence, it is possible that the two domains are related in function. In the case of CPZ, the carboxypepti-

dase domain may act directly on the Wnt protein (fig. 6, model 2). As discussed above, the CPZ catalytic domain has been shown to remove C-terminal basic amino acid residues from a series of synthetic peptides [67]. Interestingly, several Wnt proteins are predicted to contain C-terminal Lys or Arg residues, based on analysis of the cDNA sequence. For example, a C-terminal Lys residue is encoded by the genes for human Wnt-5a, human Wnt-7a, and many mouse Wnts (-3a, -5b, -7b, -10a, -10b and -11). A C-terminal Arg residue is encoded by the gene for mouse Wnt-4. However, it is not known whether the major forms of these proteins that exist in vivo contain the C-terminal basic residue, or whether it has been removed by a carboxypeptidase. If processing does occur at this site, it is not known whether this affects the biological activity. It is possible that removal of the C-terminal residue activates or inactivates the Wnt, renders it susceptible to further degradation or alters the targeting of the Wnt within the extracellular matrix. If cleavage of the C-terminal Lys or Arg by CPZ decreases the activity of the Wnt, then this effect of CPZ would be complementary to the proposed downregulation of Wnt activity by the Cys-rich domain within CPZ.

It is also possible that the carboxypeptidase domain of CPZ plays a complementary role to the frizzled homology domain by cleaving a substrate other than Wnt proteins (fig. 6, model 3). For example, during development the extracellular matrix undergoes substantial remodeling. A large number of endoproteases have been found within the extracellular matrix, and many of these are highly regulated during development. Endopeptidases, as with most enzymes, are prone to product inhibition. Although there are two products of an endopeptidase reaction, these are not usually equally potent at producing inhibition. Instead, the endopeptidases tend to cut to one side of their substrate recognition sequences, and only the product that contains the bulk of that sequence tends to produce product inhibition. Because the majority of endopeptidases cleave towards the C-terminal side of their substrate recognition sequence, a carboxypeptidase processing step is usually sufficient to relieve the product inhibition. Thus, the role of CPZ may be to enhance the activity of the endoproteases that reside in, and act on the extracellular matrix. Further studies are needed to test the various hypotheses regarding CPZ function. The high degree of conservation of this protein from rat to human argues that it plays an important biological role.

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