PCOLCE2 Encodes a Functional Procollagen C-Proteinase Enhancer (PCPE2) That Is a Collagen-binding Protein Differing in Distribution of Expression and Post-translational Modification from the Previously Described PCPE1*

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The procollagen COOH-terminal proteinase enhancer (PCPE) is a glycoprotein that binds the COOH-terminal propeptide of type I procollagen and potentiates its cleavage by procollagen C-proteinases, such as bone morphogenetic protein-1 (BMP-1). Recently, sequencing of a human expressed sequence tag, which maps near the primary angle glaucoma region on chromosome 3q21, showed it to encode a novel protein with only 43% identity with PCPE but with a similar domain structure. Here we show this novel protein to be a functional procollagen COOH-terminal proteinase enhancer with activity comparable with that of PCPE and thus propose the designations PCPE2 and PCPE1, respectively. PCPE2 is shown to have a much more limited distribution of expression than does PCPE1, with strong expression in nonossified cartilage in developing tissues and at high levels in the adult heart. PCPE2 is shown to be a glycoprotein that differs markedly in the nature of its glycosylation from that of PCPE1. PCPE2 is also shown to have markedly stronger affinity for heparin than PCPE1, which may account for higher affinities for cell layers. Unexpectedly, both PCPE1 and PCPE2 were found to be collagen-binding proteins, capable of binding at multiple sites on the triple helical portions of fibrillar collagens and also capable of competing for such binding with procollagen C-proteinases. The latter observations may provide insights into the ways PCPEs affect the kinetics of the C-proteinase reaction and into the physical interactions that occur between procollagen C-proteinases and their substrates.

Procollagen precursors of the major fibrillar collagens I–III contain N- and C-propeptides1 that are cleaved to yield the mature triple helical monomers capable of forming fibrils (1). The C-propeptides are cleaved by procollagen C-proteinase (PCP) activity provided by bone morphogenetic protein-1 (BMP-1) (2, 3) and by other closely related metalloproteinases (2, 3, 4). PCP activity is potentiated by the 55-kDa glycoprotein procollagen C-proteinase enhancer (PCPE) and by 36- and 34-kDa proteolytic fragments of PCP (5, 6). PCPE contains two NH2-terminal CUB domains (7), motifs thought to be involved in protein-protein interactions and found in various proteins with roles in development (8). PCPE also contains a COOH-terminal NTR domain (7) that has homology with NH2-terminal domains of tissue inhibitors of metalloproteinases and with COOH-terminal domains of netrins, complement components C3, C4, and C5, and secreted frizzled-related proteins (9). The 36- and 34-kDa PCPE fragments retain full PCP-enhancing activity and, like the full-length 55-kDa form, bind type I procollagen C-propeptides (5, 6). Since the 36- and 34-kDa forms contain little or no sequences other than the two CUB domains (7), such abilities appear to reside exclusively in these motifs. Indeed, it has been suggested that the CUB-containing 34- and 36-kDa fragments may possess higher levels of PCP-enhancing activity than full-length PCPE (6). In contrast to activities provided by the NH2-terminal portion of PCPE, the cleaved COOH-terminal NTR domain, consistent with its homology with tissue inhibitors of metalloproteinases, has the ability to inhibit matrix metalloproteinases (10). Thus, in addition to whatever functions are provided by full-length PCPE, it may serve as a precursor from which functional NH2- and COOH-terminal products are derived. Interestingly, although these products appear to provide very different activities, both would serve to foster a net deposition of fibrous matrix. PCPE may serve additional roles, since disruption of the rat PCPE gene can result in anchorage-independent growth and loss of contact inhibition in cultured fibroblasts (11). It is not clear whether such effects on growth control may be secondary to effects on matrix deposition or whether they might represent additional PCPE functions.

A human expressed sequence tag from the 3q21-q24 chromosomal region, to which a primary open angle glaucoma locus had been mapped, was found to encode a protein with a domain structure similar to that of PCPE and 43% identity in amino acid sequence (12). However, although this novel protein is found at relatively high levels in the trabecular meshwork of

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1 The abbreviations used are: N-propeptide, amino-terminal propeptide; C-propeptide, carboxyl-terminal propeptide; PCP, procollagen C-proteinase; BMP-1, bone morphogenetic protein-1; PCPE, procollagen C-proteinase enhancer; CUB domain, complement-Uegf-BMP-1 domain; NTR domain, netrin domain; dpc, days post conception; PBS, phosphate-buffered saline; mTLL-1, mammalian Tolloid-related 1; mTLL-2, mammalian Tolloid; mTLD, mammalian Tolloid-like 2; Tsg, twisted gastrulation; dpc, days post conception; PNGase F, peptide N-glycosidase F.

2 B. M. Steiglitz and D. S. Greenspan, unpublished observations.
the eye, a screen for coding sequence mutations in FCOLCE2, the gene that encodes this novel protein, were negative, thus excluding FCOLCE2 as a primary open angle glaucoma candidate gene (12).

In the present report, we demonstrate that the protein product of FCOLCE2 is a procollagen C-proteinase enhancer, with levels of activity similar to that of PCPE. We thus suggest that it be designated procollagen C-proteinase enhancer 2 (PCPE2), with redesignation of PCPE as PCPE1. PCPE2 is shown to be a glycoprotein that differs from PCPE1 in the nature of its carbohydrate side chains. It is also shown to bind with markedly greater affinity to heparin than does PCPE1, consistent with previous observations (12) that it is more closely associated with cell layers. Analysis of a wide range of developing and adult tissues shows PCPE2 to have a distribution of expression strikingly different and much more limited than that of PCPE1. Surprisingly, both PCPE1 and PCPE2 were found to bind to the trihelical portion of fibrillar collagen and to compete in such binding with procollagen C-proteinases. Possible implications of the data are discussed.

**EXPERIMENTAL PROCEDURES**

**Isolation of Human and Mouse PCPE2 cDNA Sequences—** Human PCPE2 coding sequences, lacking only signal peptide-encoding sequences, were amplified from human placenta cDNA (Clontech) by PCR with forward primer 5'-AGTGTACCTGAGCTGAGCTCCAGGAGA- CCGTGCTTC-3' and reverse primer 5'-CGGGAGGGTTTCCAATCAGATAGCTGC-3', corresponding to nucleotides 1481–1546 of the published human PCPE2 sequence (12) (GenBank accession number AF098269) and containing an Nhel site to facilitate cloning, and reverse primer 5'-GGGGAGGGTTTCCAATCAGATAGCTGC-3', corresponding to nucleotides 1481–1506. The resulting ~1.4-kb PCR product was cloned into pGEM-T (Promega), and inserts were sequenced using SP6 and T7 primers to identify an error-free clone. To obtain novel mouse PCPE2 sequences, a ~300-bp PshAI/AlI fragment of human PCPE2 cDNA was used to screen a mouse heart cDNA 5'-STRITCH PLUS library (Clontech). As a consensus sequence for full-length mouse PCPE2 cDNA was obtained by sequencing all inserts on both strands.

**RNA Blot Analysis—** Probes for RNA blot analysis of human and mouse PCPE2 were prepared by PCR amplification using corresponding expression vectors containing full-length PCPE1 or PCPE2 sequences as templates. For NPCPE1/FLAG, the forward primer was that used for the full-length PCPE1 expression vector. The reverse primer, containing a NotI site and FLAG coding sequences, was 5'-GACAAGCGGCGCAGCTCGACGTCCTCGTCCTGTCGTCAGCTTCTTC-3'. The truncated PCPE2 product ends at amino acid residue 288, within the linker region between the NTR and second CUB domains, forming the FLAG epitope. For NPCPE2/FLAG, the forward primer was that used for the full-length PCPE2 expression vector, whereas the reverse primer, containing a NotI site and FLAG peptide coding sequences, was 5'-GACAAGCGGCGCAGCTCGACGTCCTCGTCCTGTCGTCAGCTTCTTC-3'. The truncated PCPE2 product ends at amino acid residue 287, within the linker region between the NTR and second CUB domains, followed by the FLAG epitope. NPCPE1/FLAG and NPCPE2/FLAG inserts were cloned into pCEP/Pu/BM40s, as described above for full-length inserts.

Human 293 EBNA-1 cells (Invitrogen) were maintained as described (15) and transfected using LipfectAMINE (Invitrogen). Two days post-transfection, media were replaced with complete Dulbecco's modified Eagle medium (Cellgro), containing 10% fetal bovine serum (HyClone), 1× l-glutamine (Cellgro), 250 μg/ml Geneticin (Invitrogen), and 5 μg/ml puromycin (Sigma). Cells were selected in media containing puromycin, not less than 2 weeks, and surviving cells were allowed to grow to confluence cultures in the same selective media. Confluent monolayers were washed three times with phosphate-buffered saline (PBS) and incubated 24 h in Dulbecco's modified Eagle medium containing 1× l-glutamine, 5 μg/ml puromycin, and 40 μg/ml soybean trypsin inhibitor (Sigma). Harvested media were centrifuged to remove debris, and protease inhibitors were added to final concentrations of 10 mM EDTA, 1 mM aminoenzioic acid, 1 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. Conditioned media, previously equilibrated in PBS, were applied to 5 mg of heparin-Sepharose, previously swollen, equilibrated with 0.5 g/ml puromycin, and were stored at ~70 °C until use. FLAG-tagged NPCPE1 and NPCPE2 proteins, each ~1 μg/ml, were purified from media as described (4).

**Heparin-Sepharose Chromatography—** Fifty ml of conditioned media, containing PCPE1 or -2 from 293-EBNA mass cultures were applied to a column containing 0.5 g of heparin-Sepharose (Amersham Biosciences) previously equilibrated in PBS. The column was washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and then with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl. Subsequently, protein was eluted with 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, and fractions containing PCPE1 or PCPE2, as determined by SDS-PAGE, were dialyzed extensively against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. Fractions containing PCPE1 and PCPE2 were calculated by comparing intensities of Coomasie Brilliant Blue-stained bands from serial dilutions of each sample to those of serially diluted protein standards of known concentrations.

To determine relative affinities of PCPE1 and PCPE2 for heparin, 5 ml of conditioned media from mass cultures of 293 EBNA-1 cells were applied to 5 mg of heparin-Sepharose, previously swollen, equilibrated with PBS, and poured into a 0.8 × 4-cm Poly-Prep column (Bio-Rad). Media were reapplied to the matrix four times, and columns were washed with 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl. Subsequently, protein was eluted with 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, and fractions containing PCPE1 or PCPE2, as determined by SDS-PAGE, were dialyzed extensively against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. Fractions containing PCPE1 and PCPE2 were calculated by comparing intensities of Coomasie Brilliant Blue-stained bands from serial dilutions of each sample to those of serially diluted protein standards of known concentrations.

**Enzymatic Deglycosylation and Lectin Binding Studies—** Human 293 EBNA-1 cells (Invitrogen) were maintained as described (15) and transfected using LipfectAMINE (Invitrogen). Two days post-transfection, media were replaced with complete Dulbecco's modified Eagle medium (Cellgro), containing l-glutamine (Cellgro), 250 μg/ml Geneticin (Invitrogen), and 5 μg/ml puromycin (Sigma). Cells were selected in media containing puromycin, not less than 2 weeks, and surviving cells were allowed to grow to confluence cultures in the same selective media. Confluent monolayers were washed three times with phosphate-buffered saline (PBS) and incubated 24 h in Dulbecco's modified Eagle medium containing 1× l-glutamine, 5 μg/ml puromycin, and 40 μg/ml soybean trypsin inhibitor (Sigma). Harvested media were centrifuged to remove debris, and protease inhibitors were added to final concentrations of 10 mM EDTA, 1 mM p-aminoenozioic acid, 1 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. Conditioned media, previously equilibrated in PBS, were applied to 5 mg of heparin-Sepharose, previously swollen, equilibrated with 0.5 g/ml puromycin, and were stored at ~70 °C until use. FLAG-tagged NPCPE1 and NPCPE2 proteins, each ~1 μg/ml, were purified from media as described (4).
Collagen Binding Assays—5 µl of bovine type I collagen (Vitrogen, 3 mg/ml, Cohesion Technologies) was diluted to 100 µl with PBS and combined with 200 ng of recombinant PCPE1, PCPE2, NPCPE2/FLAG, NPCPE1/FLAG, NPCPE2/FLAG, twisted gastrulation (Tsg)/C, BMP-1/FLAG, mTLD/FLAG, or mTLL-1/FLAG. Preparation and purification of Tsg/C, BMP-1/FLAG, mTLD/FLAG, and mTLL-1/FLAG have been described (4, 18). For polyclonal antibodies against full-length PCPE1, a previously described 1570-bp PCR product (10) was subcloned between BamHII and HindIII sites of pRSET A (Invitrogen); transfected bacteria were lysed in 50 mM NaH2PO4, pH 8.0, 20 mM Tris-HCl, 6 mM urea, 100 mM NaCl; and the His-tagged PCPE1 was purified on TALON metal affinity concentrator (Millipore Corp.). Purity of the sample was verified by SDS-PAGE and E-Zinc staining (Pierce).

Collagen Binding assays—5 µl of bovine type I collagen (Vitrogen, 3 mg/ml, Cohesion Technologies) was diluted to 100 µl with PBS and combined with 200 ng of recombinant PCPE1, PCPE2, NPCPE1/FLAG, NPCPE2/FLAG, twisted gastrulation (Tsg)/C, BMP-1/FLAG, mTLD/FLAG, or mTLL-1/FLAG. Preparation and purification of Tsg/C, BMP-1/FLAG, mTLD/FLAG, and mTLL-1/FLAG have been described (4, 18). NPCPE1/FLAG and NPCPE2/FLAG were purified as described for other FLAG-tagged proteins (4, 18). For polyclonal antibodies against full-length PCPE1, or antibodies directed against COOH-terminal FLAG (4) or protein C (18) epitopes. To examine simultaneous binding of PCPEs and mTLL-1, 3 mTLL-1/FLAG or E-Zinc protein staining for PCPE1 or PCPE2 subjected to SDS-PAGE, and processed for immunoblot detection of complete adjuvant.

In Vivo Procollagen Cleavage Assays—Recombinant 3H-labeled type II procollagen was prepared as previously described (4), and 400 ng were incubated 30 min at 37 °C alone or in the presence of an equimolar amount (~133 ng) of recombinant PCPE1 or PCPE2 in 50 ml Tris-HCl, pH 7.5, 150 mM NaCl. Recombinant mammalian Tolloid-like 1 (mTLL-1), with a COOH-terminal FLAG epitope, was prepared and purified as previously described (4), and 30 ng were added to each reaction, with 0.1 mM CaCl2 to bring the final concentration to 5 mM CaCl2. Samples were returned to 37 °C, and reactions were quenched at the indicated times by adding 10× concentrating SDS-PAGE sample buffer containing 2-mercaptoethanol and boiling for 5 min. Samples were subjected to SDS-PAGE on 5% acrylamide gels, which were treated with ENHANCE (DuPont) and exposed to film.

RESULTS

Differential Expression of PCOLCE2 and PCOLCE1 in Adult and Developing Human Tissues—To gain insights into possible differences/similarities in the in vivo roles of PCOLCE1 and PCOLCE2 gene products, we first compared distributions of expression of the two genes in a dot blot array of poly(A)+ RNA from a broad range of adult and fetal human tissues. As can be seen (Fig. 1A), both PCOLCE1 and -2 are highly expressed in adult human heart. This is particularly evident for PCOLCE2, for which high levels of expression are mostly confined to the aorta and portions of the heart (Fig. 1A, column 4) but which otherwise appears to have a much more limited distribution of expression than PCOLCE1 (Fig. 1). The other only tissues in which high levels of PCOLCE2 expression are seen are pituitary gland, bladder, mammary gland, and trachea. It should be noted that the latter is the only representative of nonskin tissue on the dot blot array, since trachea contains nonossified cartilaginous rings. Thus, high expression of PCOLCE2 in adult trachea suggests that the high expression of Pcolce2 localized to nonossified cartilage in developing bone (see below) may persist in nonossified cartilage in the adult.

The dot blot array shows high levels of PCOLCE1 expression to be more broadly distributed than was observed for PCOLCE2, in both adult and fetal human tissues. Thus, for example, levels of PCOLCE1 expression throughout the digestive tract (Fig. 1A, column 5, and spots 6A–6C) are similar to levels observed in heart, and, as previously reported (13), PCOLCE1 expression is at particularly high levels in uterus. Interestingly, PCOLCE1 expression is also high in various fetal soft tissues, whereas PCOLCE2 expression is not, suggesting important roles for the former but not the latter in the development of these soft tissues. PCOLCE2 is not even expressed at high levels in fetal heart, despite the high levels of PCOLCE2 expression found in the adult organ. Thus, PCOLCE2 may play a greater role in adult than in developing heart.

Northern blot analysis of poly(A)+ RNAs from a smaller set of human adult tissues (Fig. 1B) gave results consistent with those seen in the dot blot arrays; high levels of expression of PCOLCE1 and even higher levels of PCOLCE2 are detected in the heart, with lower levels of expression of both genes in other soft tissues and no detection of expression of PCOLCE2 and barely perceptible expression of PCOLCE1 in brain. We have previously used a Northern blot of human adult poly(A)+ RNAs from tissues other than those represented in the blot of Fig. 1B to show high expression of PCOLCE1 in the uterus (13). For the present study, that same blot was stripped and rehybridized to a PCOLCE2 probe (Fig. 1C), yielding results that confirm that PCOLCE2, unlike PCOLCE1, is not expressed at high levels in this tissue in humans.

Collagen Binding Assays—5 µl of bovine type I collagen (Vitrogen, 3 mg/ml, Cohesion Technologies) was diluted to 100 µl with PBS and combined with 200 ng of recombinant PCPE1, PCPE2, NPCPE1/FLAG, NPCPE2/FLAG, twisted gastrulation (Tsg)/C, BMP-1/FLAG, mTLD/FLAG, or mTLL-1/FLAG. Preparation and purification of Tsg/C, BMP-1/FLAG, mTLD/FLAG, and mTLL-1/FLAG have been described (4, 18).NPCPE1/FLAG and NPCPE2/FLAG were purified as described for other FLAG-tagged proteins (4, 18). After incubation for 3 h at 37 °C, collagen was precipitated by centrifuging 5 min at 12,000 × g, 4°C. SDS-PAGE sample buffer was added to supernatants, whereas pellets were washed with PBS and resuspended in SDS-PAGE sample buffer. Supernatants and pellets were analyzed by SDS-PAGE and Western blot analysis using PCPE2 antibodies, antibodies directed against full-length PCPE1, or antibodies directed against COOH-terminal FLAG (4) or protein C (18) epitopes. To examine simultaneous binding of PCPEs and mTLL-1, 3 mTLL-1/FLAG or E-Zinc protein staining for PCPE1 or PCPE2.

Rotary Shadowing and Electron Microscopy—250 ml of media containing recombiant PCPE2 were applied to a 5-ml heparin-Sepharose column, which was washed with 50 ml of 150 mM NaCl, 20 mM sodium phosphate, pH 7.4, and eluted with 50 ml of a 0.15–1 M NaCl gradient in 20 mM sodium phosphate, pH 7.4. Fractions containing PCPE2, designated PCPE2/FAGE, were pooled. Following dialysis against 50 mM NaCl, 50 mM sodium phosphate, pH 7.0, the sample was applied to a 5-ml SP Sepharose column (Amersham Biosciences), washed with 25 ml of 50 mM sodium phosphate, pH 7.0, and eluted with 25 ml of a 0–0.5 M NaCl gradient in 50 mM sodium phosphate, pH 7.0. Fractions containing PCPE2 were pooled, concentrated to 0.5 ml, and exchanged into 150 mM NaCl, 50 mM sodium phosphate, pH 7.0, using an Ultrafree-4 concentrator (Millipore Corp.). Purity of the sample was verified by SDS-PAGE and E-Zinc staining (Pierce).

One part 4.9 µM PCPE2 was mixed with one part 0.7 µM type II procollagen (kind gift of Dr. Magnus Honig) in 0.1 µM (NH4)2CO3 buffer containing 1 mM MgCl2. The mixture was incubated for 15 h at 4 °C. Samples were diluted with glycerol to a final concentration of 70%. 100 µl of the sample solution was sprayed with an airbrush onto freshly cleaved mica. Rotary shadowing was performed in a Balzers BAE 250 evaporator as described previously (19). Replicas were observed using a Philips EM 410LS TEM, calibrated using a carbon-grating replica (Pul- larn 100/21). Images were taken at a final magnification of 145,000 ×. The Bioquant program (R&M Biometrics, Inc.) was used for quantitating binding events. Type I procollagen molecules with at least one bound PCPE2 were measured on a digitizing hydroip. Individual binding events were included in the data analysis only if the entire procollagen molecule and the bound PCPE2 were clearly visible. Each binding event was measured by starting at the COOH-terminal end of the type I procollagen at the base of the globular domain and continuing to the middle of the binding spot. A total of 352 binding events were counted. Binding events were then binned for every 10 nm along the collagen strand. The percentage of number of events in each bin over total events counted was calculated and plotted against the length of the collagen strands. The dot blot array shows high levels of PCOLCE1 expression to be more broadly distributed than was observed for PCOLCE2, in both adult and fetal human tissues. Thus, for example, levels of PCOLCE1 expression throughout the digestive tract (Fig. 1A, column 5, and spots 6A–6C) are similar to levels observed in heart, and, as previously reported (13), PCOLCE1 expression is at particularly high levels in uterus. Interestingly, PCOLCE1 expression is also high in various fetal soft tissues, whereas PCOLCE2 expression is not, suggesting important roles for the former but not the latter in the development of these soft tissues. PCOLCE2 is not even expressed at high levels in fetal heart, despite the high levels of PCOLCE2 expression found in the adult organ. Thus, PCOLCE2 may play a greater role in adult than in developing heart.

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Characterization of Murine Pcolce2 Coding Sequences and Examination of the Differential Expression of Pcolce2 and Pcolce1 in Mouse Development—We have previously provided full-length cDNA sequences for the PCPE1 product of the human PCOLCE1 and mouse Pcolce1 genes (7). To enable comparative analyses of the expression profiles of the mouse Pcolce1 and Pcolce2 genes in the present report, we have isolated cDNA clones encoding the product of Pcolce2, such that murine probes for both Pcolce2 and Pcolce1 would be available for Northern blot analysis of adult mouse tissues and for in situ hybridization analyses of expression of the two genes during mouse development. The full-length amino acid sequence for the Pcolce2 product is presented in Fig. 2. The molecular weight calculated for the 414-amino acid mouse protein deduced from the cDNA sequence is 45,398 (pl 9.28), whereas cleavage of the putative signal peptide (Fig. 2) would produce a 393-amino acid secreted protein with a predicted molecular weight of 43,199 (pl 9.25). Although the secreted form of the protein product of the human PCOLCE2 gene has previously been predicted to have a molecular weight of ~46,800, based on the cDNA sequence (12), we have calculated the molecular weight of the full-length 415-amino acid precursor of the human protein to be only 45,716 (pl 8.81) and the mature secreted form, minus predicted signal peptide sequences, to have a molecular weight of 43,278 (pl 8.67). Thus, products of the human PCOLCE2 and mouse Pcolce2 genes are extremely conserved in size, differing in length by a single amino acid in the signal peptide. This is in contrast to human and mouse PCPE1, whose secreted mature forms, 424 and 444 amino acids, respectively, differ by 20 amino acids in the linker region between the CUB2 and NTR domains (7). The linker region is, therefore, relatively extended and unconserved in human and mouse PCPE1 but shortened and highly conserved in the PCOLCE2 and Pcolce2 gene products. Notably, an RGD sequence previously reported in the human PCOLCE2 gene product and suggested as a possible cell attachment site (12) is not conserved in the murine Pcolce2 product. In contrast, a single potential site for N-linked glycosylation in the human PCOLCE2 product is conserved in mouse Pcolce2 (Fig. 2), suggesting functional relevance.

Probes prepared from Pcolce2 cDNA enabled examination of Pcolce2 expression in mouse development. Initial insights into the temporal expression of Pcolce2 during murine development were obtained using a Northern blot of poly(A)⁺ RNA from a...
mouse PCPE (of signal peptide sequences. The mouse and human versions of PCOLCE2 in the two species are shaded, and the arrowsheads mark the starts of the two CUB domains (CUB1 and CUB2) and of the COOH-terminal netrin-like domain (NTR). The mouse PCOLCE2 sequence KGD, which corresponds to an RGD sequence in human PCOLCE1. In contrast, at 13.5 dpc, PCOLCE1 expression appears to be the inverse of that of PCOLCE2, since the former is expressed in Meckel’s cartilage, whereas expression of the latter is excluded from Meckel’s cartilage but is at high levels in an adjacent site of ossification. Cartilage surrounding the nasal passages (e.g., cartilage primordia of the turbinates) and is especially evident in the inominate bone (pelvis) in which both PCOLCE2 expression and alcin blue staining are seen to be excluded only from a central region of ossification. In the jaw, as in other portions of 15.5-dpc embryos, PCOLCE2 expression appears to be the inverse of that of PCOLCE1, since the former is expressed in Meckel’s cartilage, whereas expression of the latter is excluded from Meckel’s cartilage but is at high levels in an adjacent site of ossification within the mandible and within the primordium of a lower incisor (Fig. 4).

Comparison of the Properties of Recombinant Protein Products of PCOLCE1 and PCOLCE2: Expression and Heparin Binding—To enable characterization of the biochemical properties of the PCOLCE2 protein product and comparison with the PCOLCE1 product (PCPE), recombinant versions of the two proteins were separately expressed in transfected 293-EBNA cells, which typically secreted ~5 μg/ml of the former and ~8 μg/ml of the latter into conditioned media. Each recombinant protein represented >50% of total protein in conditioned media, with the vast majority of additional proteins in samples consisting of added soybean trypsin inhibitor and residual bovine serum albumin from serum. Preliminary binding assays with various affinity resins found both recombinant proteins, but not bovine serum albumin or soybean trypsin inhibitor, to bind heparin. Thus, heparin affinity chromatography was used to concentrate and enrich the two recombinant proteins from conditioned media. While the current study was in progress, Moschcovich et al. (20) reported that recombinant PCPE prepared from insect cell cultures binds to heparin and elutes in a linear 0.15–0.75 NaCl gradient at between 0.4 and 0.5 M NaCl. In the present report, we found recombinant PCPE prepared in mammalian 293-EBNA cell cultures to elute from heparin-Sepharose primarily between 0.3 and 0.4 M NaCl, and ~95% of PCPE or ~95% of the latter into conditioned media. Each recombinant protein represented >50% of total protein in conditioned media, with the vast majority of additional proteins in samples consisting of added soybean trypsin inhibitor and residual bovine serum albumin from serum. Preliminary binding assays with various affinity resins found both recombinant proteins, but not bovine serum albumin or soybean trypsin inhibitor, to bind heparin. Thus, heparin affinity chromatography was used to concentrate and enrich the two recombinant proteins from conditioned media. While the current study was in progress, Moschcovich et al. (20) reported that recombinant PCPE prepared from insect cell cultures binds to heparin and elutes in a linear 0.15–0.75 NaCl gradient at between 0.4 and 0.5 M NaCl. In the present report, we found recombinant PCPE prepared in mammalian 293-EBNA cell cultures to elute from heparin-Sepharose primarily between 0.3 and 0.4 M NaCl, although significant quantities of the protein eluted at lower NaCl concentrations or were found in the wash (not shown). In contrast, most of the PCOLCE2 product eluted at between 0.4 and 0.7 M NaCl, with significant amounts of material eluting at even higher NaCl concentrations, thus showing this protein to bind heparin with markedly greater affinity than PCPE (not shown). Proteins in fractions eluted from heparin-Sepharose and used for further experiments consisted of >95% PCPE or the PCOLCE2 product, as estimated by analysis of samples by SDS-PAGE and Coomassie Blue staining.

Fig. 2. Alignment of murine Pocle2 (mur2) amino acid sequences with those of human PCOLCE2 (hum2) and human and mouse PCPE (hum1 and mur1). Cysteines and consensus sequences for N-linked glycosylation are boxed, residues identical in PCPE and Pocle2/PCOLCE2 in the two species are shaded, and the arrowsheads mark the starts of the two CUB domains (CUB1 and CUB2) and of the COOH-terminal netrin-like domain (NTR). The mouse Pocle2 sequence KGD, which corresponds to an RGD sequence in human PCOLCE1. In contrast, at 13.5 dpc, PCOLCE1 expression appears to be the inverse of that of PCOLCE2, since the former is expressed in Meckel’s cartilage, whereas expression of the latter is excluded from Meckel’s cartilage but is at high levels in an adjacent site of ossification within the mandible and within the primordium of a lower incisor (Fig. 4).

Comparison of the Properties of Recombinant Protein Products of PCOLCE1 and PCOLCE2: Expression and Heparin Binding—To enable characterization of the biochemical properties of the PCOLCE2 protein product and comparison with the PCOLCE1 product (PCPE), recombinant versions of the two proteins were separately expressed in transfected 293-EBNA cells, which typically secreted ~5 μg/ml of the former and ~8 μg/ml of the latter into conditioned media. Each recombinant protein represented >50% of total protein in conditioned media, with the vast majority of additional proteins in samples consisting of added soybean trypsin inhibitor and residual bovine serum albumin from serum. Preliminary binding assays with various affinity resins found both recombinant proteins, but not bovine serum albumin or soybean trypsin inhibitor, to bind heparin. Thus, heparin affinity chromatography was used to concentrate and enrich the two recombinant proteins from conditioned media. While the current study was in progress, Moschcovich et al. (20) reported that recombinant PCPE prepared from insect cell cultures binds to heparin and elutes in a linear 0.15–0.75 NaCl gradient at between 0.4 and 0.5 M NaCl. In the present report, we found recombinant PCPE prepared in mammalian 293-EBNA cell cultures to elute from heparin-Sepharose primarily between 0.3 and 0.4 M NaCl, although significant quantities of the protein eluted at lower NaCl concentrations or were found in the wash (not shown). In contrast, most of the PCOLCE2 product eluted at between 0.4 and 0.7 M NaCl, with significant amounts of material eluting at even higher NaCl concentrations, thus showing this protein to bind heparin with markedly greater affinity than PCPE (not shown). Proteins in fractions eluted from heparin-Sepharose and used for further experiments consisted of >95% PCPE or the PCOLCE2 product, as estimated by analysis of samples by SDS-PAGE and Coomassie Blue staining.
Characterization of the Differential Glycosylation of PCPE and the PCOLCE2 Product—We found recombinant PCPE and the PCOLCE2 product to migrate on SDS-polyacrylamide gels with apparent molecular weights of 50,000 and 48,000, respectively, somewhat greater than the 45,523 Mr for PCPE and 43,278 Mr for the PCOLCE2 product predicted by amino acid sequences of the mature secreted forms. It has previously been shown by Kessler and colleagues (5, 6) that PCPE binds concanavalin A, thus identifying it as a glycoprotein. However, the nature of PCPE glycosylation has not been characterized; nor has the possibility of posttranslational modification of the PCOLCE2 product been explored. Toward the end of determining the nature of glycosylation of PCPE and the PCOLCE2 product, each protein was treated with a mixture of the five enzymes PNGase F, endo-O-glycosidase, sialidase A, β-(1–4)-galactosidase, and glucosaminidase. As can be seen, treatment with this mix of enzymes increases the mobility of each protein upon SDS-PAGE, such that each apparent molecular weight is similar to the Mr predicted by the corresponding amino acid sequence (Fig. 5A). Thus, both are glycoproteins. To elucidate the nature of its glycosylation, the PCOLCE2 product was separately incubated with each enzyme from the mixture. PNGase F, which removes virtually all N-linked oligosaccharides from glycoproteins, had no apparent effect on mobility (Fig. 5B). Thus, despite conservation of the potential N-linked glycosylation site Asn-Met-Ser in the NTR domain of both human PCOLCE2 and murine Pcolce2 protein products, this protein is not likely to contain N-linked oligosaccharides. Sialidase A (sialidase from Arthrobacter ureafaciens) removes mono-, di-, and trisialyl residues, the most commonly occurring modifications, from Galβ(1–3)GalNAc O-linked cores. Treatment of the PCOLCE2 product with sialidase A produced a marked increase in electrophoretic mobility (Fig. 5B). Endo-O-glycosidase (endo-α-N-acetylgalactosaminidase) removes Galβ(1–3)-GalNAc cores from serine and threonine residues, but only after modifying monosaccharides have first been removed by exoglycosidases. Treatment of the PCOLCE2 product with both sialidase A and endo-O-glycosidase produced an additional increase in electrophoretic mobility (Fig. 5B), although the latter enzyme alone produced no change in PCPE2 electrophoretic mobility (not shown). Thus, the PCOLCE2 product is decorated with sialylated Galβ(1–3)GalNAc O-linked cores. β-(1–4)-galactosidase and glucosaminidase, which remove less common modifying β-(1, 4)-linked galactose and β-(1–6)-linked N-acetylgalcosamine residues from Galβ(1–3)GalNAc O-linked cores, had no effect on PCOLCE2 product electrophoretic mobility (not shown) when either was used alone or in combination with the other enzymes, indicating the absence of modifying β-(1, 4)-linked galactose and β-(1–6)-linked N-acetylgalcosamine residues on Galβ(1–3)GalNAc O-linked cores. As expected from the above results, the lectin peanut agglutinin, which binds unsubstituted Galβ(1–3)GalNAc O-linked cores but not those attached to modifying saccharides, binds PCOLCE2 product treated with sialidase A alone but not untreated PCOLCE2 product or PCOLCE2 product treated with both sialidase A and endo-O-glycosidase (Fig. 5C). Also as expected, β-(1–4)-galactosidase and glucosaminidase had no effect on peanut agglutinin binding by PCOLCE2 product (not

Fig. 3. Expression domains of the Pcolce2 and Pcolce1 genes in 10.5- and 13.5-dpc mouse embryos. Photomicrographs are shown for sagittal sections of 10.5-dpc (A) and 13.5-dpc (B) mouse embryos characterized by in situ hybridization with Pcolce2 or Pcolce1 antisense riboprobes. BA, branchial arches; CL, clavicle; FE, femur; IN, intestine; RI, ribs; ST, stomach; UV, umbilical vessels.
shown). Collectively, the results thus indicate glycosylation of the PCOLCE2 product to consist solely of sialylated Galβ(1-3)GalNAc O-linked cores.

In contrast to the PCOLCE2 product, treatment of PCPE with PNGase F alone resulted in mobility shifts to produce what appears to be a triplet of bands; one of which had a mobility similar to that of untreated PCPE, one of which had a mobility similar to that predicted by the amino acid sequence of the secreted form of PCPE, and the other of which had a mobility intermediate between those of the other two bands (Fig. 5D). Thus, PCPE, which contains two potential sites for N-linked glycosylation (7), differs from the PCOLCE2 product in that it contains N-linked oligosaccharides. Treatment of PCPE with sialidase A alone resulted in a mobility shift to produce two bands; one of which had a mobility similar to that of untreated PCPE and one of which had a mobility similar to that predicted by the amino acid sequence of secreted PCPE. Interestingly, treatment with sialidase A plus endo-O-glycosidase produced no additional mobility changes (Fig. 5D). In addition, enzymes β(1-4)-galactosidase and glucosaminidase had no effects on PCPE mobility, either when used alone or in combination with the other enzymes (not shown). Thus, although oligosaccharides on PCPE are decorated with sialyl residues, Galβ(1-3)GalNAc O-linked cores are absent. However, although Galβ(1-3)GalNAc O-linked cores are absent, PCPE treated with sialidase A, alone or in combination with other enzymes, still binds peanut agglutinin (Fig. 5E). Thus, in addition to N-linked oligosaccharides, PCPE is also decorated with complex O-linked oligosaccharides (e.g. containing α-fucose or α-galactose modifications). The latter are probably heterogeneously sialylated, accounting for the varying mobilities of PCPE forms produced upon treatment of PCPE with PNGase F alone (Fig. 5D). The varying mobilities of PCPE forms produced upon treatment with sialidase A alone suggest heterogeneity in N-linked glycosylation of the recombinant protein.

The PCOLCE2 Product Is a Procollagen C-Proteinase Enhancer—Although the PCOLCE2 product has a domain structure similar to that of PCPE, the two proteins share only 43% identity in their amino acid sequences (7, 12). This divergence of sequences, plus observed differences in distributions of expression (above), suggested the possibility of divergent functions for the two proteins. To determine whether the PCOLCE2 product is, like PCPE, a procollagen C-proteinase enhancer, similar amounts of the two proteins were incubated with human type II procollagen in the presence or absence of the procollagen C-proteinase BMP-1. As can be seen (Fig. 6A), both PCPE and the PCOLCE2 product enhance cleavage of the type II procollagen C propeptide by BMP-1, to produce pNε1(II) chains, whereas neither has intrinsic procollagen C-proteinase activity. Thus, the PCOLCE2 product (and, by inference, the product of the murine Pcolce2 gene) is a procollagen C-proteinase enhancer. This justifies designating products of PCOLCE2 and Pcolce2 as PCPE2, with redesignation of PCPE as PCPE1.

The protease mTLL-1 has PCP activity in vitro but at lower levels than does BMP-1 (4). In an attempt to compare the levels of PCP-enhancing activity of PCPE2 and PCPE1, similar amounts of the two proteins were incubated with human type II procollagen in the presence or absence of a small amount of mTLL-1, and the extent of PCP enhancement that occurred was monitored at three time points over the course of 24 h. As can be seen (Fig. 6B), levels of PCP enhancement are similar for the two proteins at 1, 6, and 24 h. Thus, PCPE2 and PCPE1 appear to have similar levels of PCP-enhancing activity.

Binding of Exogenous PCPE1 to Tissues—The binding of PCPE1, and stronger binding of PCPE2, to heparin (see above) suggested that these proteins might normally bind heparan sulfate proteoglycans in tissues. Proteins that normally bind heparan sulfate proteoglycans in vivo can, when added exog-
A, or sialidase A plus endo-
PCOLCE2
trophoretic patterns, visualized by zinc staining, are compared for
/H9252
O
proteins with a mixture of the five enzymes PNGase F, endo-
unpublished observations.

PCPE1 (see
able to detect their cognate proteins in this type of assay. In
(12) antibodies raised against short synthetic peptides were
PCPE2 antibodies. Neither anti-PCPE1 (10) or anti-PCPE2
we attempted to detect them using anti-PCPE1 and anti-
icubated on frozen sections of a 16.5-dpc mouse embryo, and
ent manner, recombinant PCPE1 and PCPE2 were separately
might bind tissues in a heparan sulfate proteoglycan-depend-
(17, 21). To determine whether exogenous PCPE1 or PCPE2
incrus, binding to type I or type II collagen fibrils in the centri-
ugation pellets (Fig. 8A). Upon incubation under the same
conditions as those used above, but in the absence of collagen,
neither PCPE1 nor PCPE2 was detectable in pellets subse-
quent to centrifugation (not shown). Thus, the presence of
PCPE1 and -2 in pellets is wholly dependent upon associations
with collagen fibrils. Incubation with collagen, under the same
conditions as those used above, but in the absence of collagen,
PCPE1 was added to sections showed no signal (not shown),
demonstrating the signal to be from exogenously added, rather
than endogenous, PCPE1. However, controls in which tissue
sections were treated with heparitinase prior to incubation,
which removes heparan sulfate proteoglycans (17, 21), still
bound PCPE1 (not shown), demonstrating binding of exoge-
nous PCPE1 to be independent of heparan sulfate proteogly-
cans. The specific binding of PCPE1 to tissues rich in collagen
fibrils suggested that PCPE1 might bind to collagen or to some
collagen-associated protein(s).

PCPE1 and PCPE2 Bind Collagen—To determine whether
PCPE1 and PCPE2 are capable of binding collagen, equimolar
amounts of PCPE1 or PCPE2 were separately incubated with
an excess of type I collagen or an excess of type II collagen
under conditions in which fibrillogenesis occurs. Upon centri-
fugation of the reaction mixtures, both PCPE2 and PCPE1 are
found bound to type I or type II collagen fibrils in the centri-
fugation pellets (Fig. 8A). Upon incubation under the same
conditions as those used above, but in the absence of collagen,
PCPE1 and -2 were capable of binding collagen. Sequence
similarities suggest that PCPE1 and PCPE2 are capable of bind-
ing collagen, with PCPE1 having a higher affinity than PCPE2.
In further experiments, we compared binding of PCPE1 and PCPE2
with that of a third collagen binding protein, BMP-1. PCPE1 and
PCPE2 were incubated with BMP-1, type I collagen, or type II
collagen in the absence or presence of BMP-1, PCPE, or the
/PCOLCE2 gene product.

Fig. 5. Characterization of the glycosylation of the /PCOLCE2
product and PCPE. A. Western blot analyses using antibodies specific
for the /PCOLCE2 product or PCPE show the effects of treating the two
proteins with a mixture of the five enzymes PNGase F, endo-O-glyco-
sidase, sialidase A, β-(1–4)-galactosidase, and glucosaminidase. B, elec-
trophoretic patterns, visualized by zinc staining, are compared for
/PCOLCE2 product incubated alone or with either PNGase F, sialidase
A, or sialidase A plus endo-O-glycosidase. C, blot analysis of binding of
the lectin peanut agglutinin (PNA) to /PCOLCE2 product incubated
alone or with either sialidase A or sialidase A plus endo-O-glycosidase.
D, lectrophoretic patterns, visualized by zinc staining, are compared for
PCPE incubated alone or with either PNGase F, sialidase A, PNGase
F plus sialidase A, sialidase A plus endo-O-glycosidase, or the
three enzymes combined. E, blot analysis of binding of the lectin peanut
agglutinin (PNA) to PCPE. Samples in lanes 1–6 were treated as in
lanes 1–6 in D. Molecular masses (in kDa) are indicated for protein
standards.

PCOLCE2—Procollagen COOH-terminal Proteinase Enhancer Protein 2

Fig. 6. The /PCOLCE2 product is a procollagen C-proteinase
enhancer. A, autofluorograms are shown of type II procollagen incu-
bated in the absence (−) or presence (+) of BMP-1, PCPE, or the
/PCOLCE2 gene product. B, autofluorograms are shown of a time course
experiment in which type II procollagen is incubated for the designated
periods of time in the absence (−) or presence (+) of /TLL-1, PCPE, or the
/PCOLCE2 gene product.

3 W. N. Pappano, B. L. Allen, A. C. Rapraeger, and D. S. Greenspan,
unpublished observations.
sought to determine whether procollagen C-proteinases (PCPs), which are thought to bind procollagen C-propeptides via CUB domains (2), might also bind the type I collagen triple helix. Interestingly, BMP-1, mTLD, and mTLL-1, the three closely related metalloproteinases shown to have PCP activity (2, 4), were all found to bind type I collagen (Fig. 8C). This binding was essentially inhibited by equimolar amounts of either PCPE2 or PCPE1 (Fig. 8D).

Rotary Shadowing and Electron Microscopy—Rotary shadowing electron microscopy of PCPE2 detected multiple binding sites on mature type I collagen monomers (not shown). To better define PCPE2 triple helix binding sites, type I procollagen was used for subsequent analyses, since this enabled defining the positions of binding sites on the triple helix in respect to the easily identifiable C-propeptide. Fig. 9 shows representative micrographs of PCPE2 binding to type I procollagen. A consistent orientation of PCPE2 relative to the collagen helix was not noted. Multiple binding sites in the helical portions of single procollagen molecules were observed. The distance between the base of the COOH-terminal globular propeptide and the site where PCPE2 bound was measured for 352 procollagen-PCPE complexes. There are no well defined peaks in the histogram, although a site ~60 nm from the C-propeptide and a broad region in the center of the molecule may be favored. Measurements of PCPE2 monomers suggest a symmetrical “barbell” shaped molecule, with the globular domains on each side measuring ~4.5 nm and the helical domain between also measuring ~4.5 nm. This is similar to the shape of PCPE1 recently detected by rotary shadowing.⁴

**DISCUSSION**

In this study, we demonstrate that the PCOLCE2 gene product, with only 43% amino acid identity to PCPE, is a functional procollagen C-proteinase enhancer and suggest the designations PCPE2 and PCPE1, respectively. Clearly, however, the two PCPEs differ from one another in a number of respects. It has previously been reported that PCPE2 is more tightly associated with the cell layer than is PCPE1 and that this association might be mediated via integrin binding to an RGD sequence present in human PCPE2 but absent in human and mouse PCPE1 (12). However, provision of the mouse PCPE2 sequence in the present report demonstrates absence of the RGD site in the mouse protein, thus suggesting the human RGD site as unlikely to be essential for PCPE2 function. Nevertheless, we demonstrate that PCPE2 binds with markedly higher affinity to heparin than does PCPE1, which may contribute to higher affinities of PCPE2 for cell layers.

In the present study, we show PCPE2 to be a glycoprotein, demonstrate PCPE1 and PCPE2 to be differentially glycosylated, and characterize the nature of glycosylation for each of

⁴ S. Bernocco, B. M. Steiglitz, D. Svergun, B. Font, F. Ruggiero, S. Ricard-Blum, C. Ebel, C. Geourjon, G. Deleage, D. Eichenberger, D. S. Greenspan, and D. J. S. Hulmes, manuscript in preparation.
the two proteins. Although the same N-M-S site for potential N-linked glycosylation is conserved in mouse and human PCPE2 sequences, PCPE2 is shown to be devoid of N-linked carbohydrate side chains and, instead, is homogeneously decorated with sialylated Galβ(1–3)GalNAc O-linked cores. In contrast, PCPE1 is decorated with complex O-linked oligosaccharides (e.g., containing α-fucose or α-galactose modifications), which appear to be heterogeneously sialylated, and appears to be heterogeneously glycosylated at the two sites for potential N-linked glycosylation conserved in mice and humans. Although ramifications of the differences in glycosylation between PCPE2 and PCPE1 are not clear, these differences suggest the N-glycosylation observed in PCPE1 to be unnecessary for correct folding or for C-proteinase enhancer function, since PCPE2 has full C-proteinase enhancer function and the two proteins have highly similar shapes, as observed by rotary shadowing electron microscopy. Notably, the ability of the mixture of enzymes used in this study to induce mobility shifts in PCPE1 and PCPE2 to molecular masses consistent with the amino acid sequences of these two proteins not only shows both to be glycoproteins, but also suggests the absence of extensive additional types of post-translational modifications.

In the present report, analysis of a broad array of developing and adult mammalian tissues shows PCPE1 to be widely expressed in soft tissues and bone during development and in the adult, whereas, in contrast, expression of PCPE2 is shown to be quite limited. Interestingly, at a number of time points in development of the mouse fetus, expression of PCPE2 seemed limited to the interior nons ossified portions of cartilaginous structures while being excluded from regions of ossification. At the same developmental times, PCPE1, in addition to broad expression in soft tissues, is also expressed at high levels in skeletal elements but apparently only in perichondrium/perios teum and centers of ossification. The apparent partitioning of expression of PCPE1 and PCPE2 to the ossified and nons ossified portions of developing bone, respectively, suggests different roles for the two PCPEs in formation of this tissue.

The different, yet closely juxtaposed, domains of expression of PCPE1 and PCPE2 in developing bone begs the question of whether the two proteins freely diffuse between the different regions or whether movement of these proteins is restricted to the domains in which they are produced through binding to components of the extracellular matrix and/or cell surfaces. Both proteins were found to bind heparin, suggesting that they might bind tissue proteoglycans. However, although exogeneously added PCPE1 was found to bind specific structures in tissue sections, it did so in a heparan sulfate-independent manner. The fact that structures bound by PCPE1 (e.g., bone and tendon) are those known to have a particularly high content of fibrillar collagens suggested that PCPE1 might bind to fibril-associated proteins or to fibrillar collagen itself. In vitro binding assays confirmed that both PCPE1 and PCPE2 are collagen-binding proteins. In fact, even at concentrations as low as 10 nM, at the limits of our ability to detect PCPEs by Western blot, 100% of detectable PCPE was found bound to collagen (not shown). This suggests a maximum apparent KD of ~10 nM for PCPE binding to the collagen triple helix, close to the ~1 nM apparent KD recently calculated for binding of PCPE1 to type I procollagen by Ricard-Blum et al. (22). Thus, although PCPE1 was first isolated, in part, via its ability to bind the type I procollagen C-propeptide (5), the majority of procollagen-binding activity may derive from binding of PCPEs to the collagen triple helical domain, especially since KD values for binding of PCPE1 to the C-propeptides of procollagens I and III have been estimated at ~170 and ~370 nM, respectively (22).

Electron microscopic analysis in the current study shows

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**Fig. 9. Rotary shadowing/electron microscopic evaluation of structure and procollagen binding of PCPE2.** Upper panel, histogram of the binding events along the type I collagen strand. The numbers along the x axis indicate the distance of each binding event from the COOH-terminal end of the triple helical portion of the procollagen monomers. The events were binned every 10 nm, and the percentages of the events in each 10-nm bin over the total binding events counted are plotted along the y axis. The histogram is printed to the same scale as the micrographs. Middle panels, representative electron micrographs showing the COOH-terminal propeptide C oriented on the leftmost portion of each micrograph and one bound PCPE molecule to the right of the globular domain. Lower panel, a field of unbound PCPE2 molecules (arrows). Scale bar, 100 nm.
that PCPE2 binds at a number of sites on the collagen triple helix. Intriguingly, data herein also show the known PCPs (BMP-1, mTLD, and mTLL-1) to be capable of binding the triple helical portion of type I collagen and that such binding is prevented by the presence of equimolar amounts of PCPE1 or PCPE2. The latter result suggests that PCPEs bind with greater affinity to the collagen triple helical region than do PCPs and, moreover, that the two classes of proteins bind the same types of sites, such that binding of PCPEs can sterically hinder binding of PCPs in our in vitro assays. Thus, taken together, the data suggest that PCPEs and PCPs both use a number of low affinity binding sites on the collagen triple helix.

We propose a model in which binding to the triple helix would facilitate the respective activities of the two classes of proteins by localizing PCPEs and biosynthetic enzymes to the substrate, such that binding would not be solely dependent on the single site at which the enzymatic reaction occurs. Binding in such a way would increase the local concentration of factors required for biosynthetic processing, limiting their ability to diffuse from the procollagen substrate, and thus facilitating PCP activity at the C-propeptide cleavage site. It seems probable that in vivo levels of PCPEs would be insufficient to saturate a majority of triple helical binding sites, which may include sites on collagen fibrils as well as on procollagen molecules. Thus, in vivo, sufficient sites would remain available for localizing PCPs to procollagen substrate, ensuring enhancement rather than inhibition of PCP activity by PCPEs.

PCPE1 has previously been shown (5) to increase the maximal velocity ($V_{max}$) of the PCP reaction. The latter has been ascribed to a conformational change, induced in procollagen by binding of PCPE1 to the procollagen C-propeptide (5, 22), that facilitates procollagen cleavage by PCPs. However, in vitro observations have previously demonstrated that PCPE1 also alters the kinetics of C-proteinase activity by increasing the apparent $K_m$ (5). The observation here that PCPEs bind the collagen triple helix with higher affinity than do PCPs provides the first conceptual explanation for the effect of PCPE1 in altering the kinetics of C-proteinase activity such that the apparent $K_m$ is increased. Thus, taken together, the two models for interaction of PCPEs, with procollagen C-propeptides and triple helical sequences, may begin to explain the seemingly contradictory kinetic data that together suggest that PCPE acts to increase the maximal velocity of PCP while at the same time increasing the substrate concentration necessary for enzyme saturation.

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