Interaction Properties of the Procollagen C-proteinase Enhancer Protein Shed Light on the Mechanism of Stimulation of BMP-1*

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Procollagen C-proteinase enhancer (PCPE) is an extracellular matrix glycoprotein that binds to the C-propeptide of procollagen I and can enhance the activities of procollagen C-proteinases up to 20-fold. To determine the molecular mechanism of PCPE activity, the interactions of the recombiant protein with the procollagen molecule as well as with its isolated C-propeptide domain were studied using surface plasmon resonance (BIACore) technology. Binding required the presence of divalent metal cations such as calcium and manganese. By ligand blotting, calcium was found to bind to the C-propeptide domains of procollagens I and III but not to PCPE. By chemical cross-linking, the stoichiometry of the PCPE/C-propeptide interaction was found to be 1:1 in accordance with enzyme kinetic data. The use of a monoclonal antibody directed against the N-terminal region of the C-propeptide suggested that this region is probably not involved in binding to PCPE. Association and dissociation kinetics of the C-propeptide domains of procollagens I and III on immobilized PCPE were rapid. Extrapolation to saturation equilibrium yielded apparent equilibrium dissociation constants in the range 150–400 nM. In contrast, the association/dissociation kinetics of intact procollagen molecules on immobilized PCPE were relatively slow, corresponding to a dissociation constant of 1 nM. Finally, pN-collagen (i.e. procollagen devoid of the C-terminal propeptide domain) was also found to bind to immobilized PCPE, suggesting that PCPE binds to sites on either side of the procollagen cleavage site, thereby facilitating the action of procollagen C-proteinases.

Bone morphogenetic protein-1 (BMP-1)1 and other tolloid-related metalloproteinases (1, 2), also known as procollagen C-proteinases (PCPs), have recently been shown to be involved in the control of a variety of morphogenetic events during development and tissue repair. These include: (i) the deposition of collagen fibrils in the extracellular matrix following the processing of procollagen propeptides (3–6); (ii) dorsoventral patterning (7–10) through the cleavage of the growth factor inhibitors chordin and SOG; (iii) collagen and elastin cross-linking by the processing of the inactive precursors of lysyl oxidases (11, 12); and (iv) adhesion/migration of epithelial cells by the cleavage of laminin 5 chains (13–15). The activities of PCPs on procollagen substrates may be stimulated up to 20-fold by another glycoprotein of the extracellular matrix, procollagen C-proteinase enhancer (PCPE) (16–19), which lacks intrinsic proteinase activity. Similarly, in the case of chordin and SOG, the protein TSG or its homologues stimulates cleavage by tolloid proteinases (20, 21), thus raising the possibility that PCP processing of different substrates might be specifically regulated by distinct enhancer proteins.

Both tolloid proteinases and PCPE are multidomain glycoproteins containing multiple copies of the so-called CUB domain (22), a protein module common to several extracellular and plasma membrane-associated proteins (23–26). The N-terminal region of tolloid proteinases consists of an astacin-like zinc metalloproteinase domain (27), whereas in the C-terminal region, CUB domains are interspersed with (calcium binding) epidermal growth factor (EGF) domains (28). In PCPE and the recently identified PCPE2 (29) the N-terminal region consists of two CUB domains, whereas the C-terminal domain is homologous to the NTR domain (30), which is also found in netrins, complement proteins, and TIMPs. PCP-enhancing activity is a property of the CUB domain region of PCPE (18, 31, 32). In contrast, the NTR domain, which is released relatively easily from the rest of the molecule by proteolytic attack (17), appears to have a moderate TIMP-like inhibitory activity against ma-

1 The abbreviations used are: BMP-1, bone morphogenetic protein-1; PCP, procollagen C-proteinase; PCPE, PCP enhancer; SOG, short gastrulation; TSG, twisted gastrulation; CUB, module found in complement subcomponents (C1r/C1s, Uegf, and BMP-1); NTR, netrin-like; TIMP, tissue inhibitor of metalloproteinases; CPI, isolated C-terminal propeptide trimer from the procollagen I molecule; CPIII, isolated C-terminal propeptide trimer from the procollagen III molecule; pN-collagen, procollagen molecule lacking the C-terminal propeptide domain; SPR, surface plasmon resonance; SANPAH, 6-[4-azido-2-nitrophenylamino]hexanoate; mAb, monoclonal antibody; SPARC, secreted protein, acidic and rich in cysteine; COMP, cartilage oligomeric matrix protein; MBL, mannann binding lectin.

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trix metalloproteinases (33). Thus, different regions of PCPE may show either stimulatory or inhibitory activities to different subfamilies of metzincin metalloproteinases in the extracellular matrix. In addition, PCPE expression is implicated in the control of cell growth (34, 35), as has also been reported for TIMPs (36).

The mechanism of PCP stimulation by PCPE is unknown. From kinetic studies (1, 16), PCPE increases both the $K_{m}$ and $V_{max}$ for PCP/BMP-1 cleavage of the C-propeptide region from procollagen I. Furthermore, maximum enhancement is achieved at an equimolar ratio of PCPE to procollagen. This suggests that enhancement occurs at the level of the substrate and not the enzyme. Here we investigate the interactions of PCPE with the procollagen type I molecule as well as with the C-propeptide trimers that are released from procollagens I and III by PCP cleavage. We show by surface plasmon resonance technology that C-propeptide binding to PCPE is dependent on divalent metal binding to the C-propeptides. Chemical cross-linking indicates that PCPE binds to the C-propeptide trimer in a molar ratio of 1:1. Finally, PCPE binding to intact procollagen molecules appears to be tighter than to the isolated C-propeptide trimers, suggesting possible additional binding sites in other regions of the procollagen molecule, a conclusion supported by the binding of PCPE to procollagen molecules devoid of the C-propeptide region.

MATERIALS AND METHODS

Proteins and Antibodies—Recombinant human PCPE as well as the C-terminal propeptide trimer from procollagen III (CPII) were expressed using baculovirus systems (19, 37, 38). Procollagen I and its C-propeptide trimer domain (CPI) were purified from the culture media of chick embryo tendon fibroblasts (39) or chick embryo tendons (40), respectively. For the preparation of pN-collagen I, procollagen I was fully cleaved with highly purified recombinant BMP-1 (1) in the absence of PCPE and then separated from both liberated C-propeptides and BMP-1 by gel filtration on a 1.6 × 60-cm column of Sephacryl S200 HR (Amersham Biosciences) equilibrated with 0.4 M NaCl, 1 M urea, and 0.1 M Tris-HCl, pH 7.4 (see ref. 41). Reduced and alkylated CPIII was prepared as described (19). The binding epitopes of the monoclonal antibodies 48D34, 48B14, and 48D19 mapped to sites within residues 3–30, 31–97, and 208–245, respectively, of the CPIII polyepitide chain as described (42).

Surface Plasmon Resonance (SPR)—Binding analysis was performed using a BIAcore Upgrade system (BIAcore AB, Upsala, Sweden). PCPE was covalently coupled to CM5 sensor chips (research grade) using carbamidemethylated dextran surface was activated by the injection of a mixture of 0.2 M N-ethyl-N′-diethylaminopropylcarbodiimide and 0.05 M N-hydroxysuccinimide. PCPE (the ligand) was then injected in 10 mM maleate buffer, pH 6.0. Activation time, PCPE concentration, and contact time were adjusted according to the desired extent of immobilization. The remaining N-hydroxysuccinimide esters were blocked by the injection of 1 mM ethanolamine hydrochloride, pH 8.5. All immobilization steps were performed at a flow rate of 5 μl/min. The immobilization of CPIII was carried out similarly except that the coupling buffer was 10 mM sodium acetate, pH 5.0. Control flow cells were prepared by carrying out the coupling reaction in the presence of coupling buffer alone; these were used to obtain control sensorgrams that were subtracted from sensorgrams obtained with immobilized ligand to yield true binding responses.

Binding assays were performed at 25 °C in 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl and 0.005% (v/v) P20 surfactant (HBS-P buffer, BIAcore AB). CPI, CPII, procollagen I, and pN-collagen I were dialyzed against HBS-P buffer and then injected at several concentrations and different flow rates over immobilized PCPE. The surface was regenerated with a pulse of 2 mM guanidinium chloride when possible. Kinetic constants were calculated by nonlinear fitting to the association and dissociation curves according to the manufacturer's instructions (BIAevaluation 3.0 software). Apparent equilibrium dissociation constants ($K_{D}$) were then calculated as the ratio of $K_{D}$ to $R_{eq}$. Alternatively, $K_{D}$ values were calculated from the equilibrium resonance signal ($R_{eq}$) as a function of analyte concentration (43), $R_{eq}$ values being estimated by extrapolation to infinite time using plots of resonance signal as a function of the reciprocal of time (44). Apparent $K_{D}$ values were then calculated by nonlinear fitting to the expression $R_{eq} = R_{max}/(K_{D} + C)$, where $R_{max}$ is the maximum binding capacity of the surface and $C$ is the analyte concentration, using Kaleidagraph software.

Ligand Blotting—Calibrated by ligand blotting and autoradiography (45, 46) using nitrocellulose membranes (Schleicher and Schuell). Different concentrations of purified proteins were applied using a dot-blot apparatus. Each well was washed twice (2 × 200 μl) with 10 mM imidazole buffer, pH 6.8, containing 60 mM KCl and 5 mM MgCl2. Membranes were incubated with 4% CaCl2 (Amersham Biosciences) for 1 h at a final concentration of 2 μg/ml for 15 min, rinsed with 50% ethanol for 5 min, dried, and exposed overnight to film (BioMax MS) in a cassette with an intensifying screen. Gelsolin (gift from A. Zapun, Institut de Biologie Structurale, Grenoble, France) was used as a positive control (47), and collagen II was used as a negative control (46).

Cross-linking—These experiments were conducted in two steps. First, purified PCPE (7.3 μM) in 0.15 μM NaCl, 10 mM Hepes, pH 7.4 was incubated in the dark with a 10-fold molar excess of the extended chain length (spacer arm 18.2 Å), photocrosslinking heterobifunctional cross-linker SANPAH (Perbio Science; reactive toward amino groups) for 15 min at room temperature. A 700-fold molar excess of glycine was then added, and the incubation was continued for 15 min. At the end of the incubation period, excess reagent was eliminated by centrifugation filtration through a 1-ml column filled with Sephadex G-50 equilibrated with 0.15 μM NaCl, 5 mM Hepes, pH 7.4. Purified CPIII (0.4 μM) was then incubated for 45 min in the dark at room temperature with an ~3-fold molar excess of PCPE-SANPAH derivative (1.3 μM) in the previously described buffer supplemented with 5 mM CaCl2. Photoactivation was then induced at 312 nm for 2.5 min with a 6 watt Spectrolamp at a distance of 7 cm from the sample. After SDS-PAGE using a 3–8% gradient gel in nonreducing conditions, proteins were electrophoreted for 4 h at 4 °C. Duplicate samples were examined by Western blotting, one using anti-rPCPE rabbit polyclonal antiseraum and the other using monoclonal antibody 48D19 against CPIII (42). Control experiments were carried out in the absence of the cross-linking reagent.

RESULTS

Binding of C-propeptide Trimmers to PCPE—Interactions were studied using surface plasmon resonance (BIAcore) technology. When passed over the surface of the sensor chip, CPIII was found to bind both specifically and in a concentration-dependent manner to immobilized PCPE (Fig. 1A). As the PCPE molecule was immobilized at pH 6.0 (see "Materials and Methods"), it is possible that PCPE was immobilized mostly via the basic NTR domain (calculated pI 9.21), leaving the two acidic domains (calculated pI 4.31 and 4.77) essentially free to interact with the C-propeptide. This is supported by experiments in which native PCPE lacking the NTR domain was immobilized when no interaction with CPIII was observed (data not shown). Because PCPE enhancement of PCP/BMP-1 is known to be a property of the CUB domain region of PCPE (18, 31, 32), it is likely that immobilization in the absence of the NTR domain interferes with CPIII binding sites in the CUB domains.

The binding of CPIII to immobilized PCPE was initially fast but then slowed down without reaching saturation equilibrium (Fig. 1A). Dissociation of bound CPIII was rapid. These combined features made fitting of a kinetic model to the data, and hence the determination of reliable on- and off-rates, difficult. Using global fitting, binding curves did not fit well to the different models included in the BIAevaluation 3.0 software (1:1 Langmuir binding, bivalent analyte, heterogeneous ligand, heterogeneous analyte, conformational change) with or without mass transport. Neither the injection at a higher flow rate (30 μl/min) nor the use of a F1 sensor chip with a shorter dextran layer changed the shape of the sensorgrams. Furthermore, because equilibrium was not reached during the association phase, the direct use of Scatchard analysis to calculate the apparent equilibrium dissociation constant was not allowed. Instead, to calculate the equilibrium dissociation constant, association curves were extrapolated to infinite time using reciprocally.
obtain apparent equilibrium dissociation constants (K_D) for reactions significantly limited by mass transport and did not involve linked nanomolar units). This suggests that the interaction was not significant (see Materials and Methods) as well as maximum binding capacities (R_max). RU, resonance unit.

rocal plots in order to estimate the equilibrium binding value R_eq obtained by extrapolation to infinite time (see "Materials and Methods") versus analyte concentration, used to determine as for CPIII. As also shown in Fig. 1B, nonlinear regression gave an apparent K_D of 169 ± 23 nM and R_max of 637 ± 11 resonance units for the interaction of CPI with PCPE.

Specific, concentration-dependent binding of CPI to CPIII could also be demonstrated in the reverse manner by injecting the whole PCPE molecule over immobilized CPIII (Fig. 2). Unlike the results with immobilized PCPE (Fig. 1A) however, these data were obtained with relatively large amounts of immobilized ligand, a condition that precluded kinetic analysis because satisfactory fitting of the association phase was not possible with the BIAevaluation 3.0 software, apparent K_D values were calculated from estimated equilibrium values determined as for CPIII. As also shown in Fig. 1B, nonlinear regression gave an apparent K_D of 169 ± 23 nM and R_max of 637 ± 11 resonance units for the interaction of CPII with PCPE. In summary, the above results show that binding of the procollagen C-propeptide trimers (types I or III) to PCPE is of moderate affinity, with K_D values in the range 150–400 nM.

Characterization of the CPIII-PCPE Interaction—CPIII bound to immobilized PCPE whether the surface was prepared in the presence or absence of 5 mM CaCl_2. Furthermore, CPIII binding was unaffected by pretreatment of the PCPE surface with HBS-P buffer containing 10 mM EDTA followed by re-equilibration in HBS-P. In contrast, when CPIII was freed of bound divalent metal cations by extensive dialysis against HBS-P containing 10 mM EDTA and then redialyzed against HBS-P alone before injection over PCPE, the binding of CPIII to immobilized PCPE was abolished (Fig. 3). The addition of 2 mM CaCl_2, MnCl_2, or MgCl_2 restored the binding (Fig. 3). These data suggest that divalent metal cation binding to CPIII is essential for binding to immobilized PCPE.

To confirm the results obtained using SPR analysis, we tested directly the binding of calcium to CPIII by the ligand blotting technique (45). Collagen I, CPI, and CPIII all showed a distinct affinity for 45Ca unlike PCPE, which did not bind 45Ca significantly under the experimental conditions used (Fig. 4). As expected (46), collagen II did not bind 45Ca, whereas gelsolin, a calcium binding protein used as a positive control (47), did.

The extent of binding of CPIII to immobilized PCPE was similar in the presence of 0.15 M, 0.3 M, and 0.45 M NaCl. Furthermore, CPIII bound PCPE to a similar extent in the absence or presence of 5 mM CaCl_2. MnCl_2, or MgCl_2 restored the binding (Fig. 3). These data suggest that divalent metal cation binding to CPIII is essential for binding to immobilized PCPE.

To identify potential PCPE binding sites within the C-propeptide domain, we used monoclonal antibodies directed against different regions of CPIII (42). Affinity-purified monoclonal antibodies (150 μg/ml) were pre-incubated at room temperature for 70 min with CPIII (150 μg/ml), and then the mixture was injected over immobilized PCPE. Using mAb 48SD34 directed against an epitope within the 30 N-terminal residues of the CPIII polypeptide chain, CPIII continued to binding or binding of heterogeneous analyte to a single ligand).
Fig. 3. CPIII binding to immobilized PCPE is dependent on divalent metal cations. CPIII (4.4 μM), dialyzed extensively versus HBS-P containing 10 mM EDTA and then re-dialyzed against HBS-P alone, was injected (15 μl/min for 4 min) over an immobilized PCPE surface (2739 resonance units), which was also pre-treated with HBS-P containing 10 mM EDTA and then re-equilibrated in HBS-P. Under these conditions (---), essentially no specific binding was detected. In contrast, when divalent metal cations were added to the same CPIII sample in HBS-P to final concentrations of 2 mM CaCl2 (-----), 2 mM MnCl2 (---) or 2 mM MgCl2 (-----) binding to the same immobilized PCPE surface was observed. Nonspecific binding to the control surface has been subtracted.

Fig. 4. Calcium binding to the procollagen C-propeptide domain. Approximately equimolar amounts of the following proteins were applied to the nitrocellulose membrane in HBS-P buffer: Procollagen I (Procoll, 20 μg) (a); CPI (5 μg) (b); CPIII (5 μg) (c); PCPE (2.5 μg) (d); collagen II (20 μg) (e); gelsolin (5 μg) (f); and HBS-P buffer (g) alone. Following incubation with 45CaCl2, binding was visualized by autoradiography as described under “Materials and Methods.”

bind to immobilized PCPE, but the SPR signal was greater than with CPIII alone (Fig. 5). No antibody binding was observed in the absence of CPIII. These results indicated that the mAb-CPIII complex bound to PCPE, thus generating an increased SPR signal, and suggested that the N-terminal region of CPIII did not participate in PCPE binding. Results using mAbs directed against other regions of CPIII were also found to bind specifically to immobilized PCPE (4044 resonance units) for 4 min at a flow rate of 15 μl/min. RU, resonance unit.

Fig. 5. Monoclonal antibody recognizing the N-terminal region of CPIII does not prevent CPIII binding to immobilized PCPE. CPIII (150 μg/ml), preincubated in HBS-P with mAb 48D34 (150 μg/ml) directed against the N-terminal part of CPIII (-----), mAb 48D34 alone (150 μg/ml) in HBS-P (----), or CPIII alone (150 μg/ml) in HBS-P (-----), was injected over immobilized PCPE (4044 resonance units) for 4 min at a flow rate of 15 μl/min.
C-propeptide domains of the procollagen I and III have been described (50, 51). Using PROSCAN (52), we found that the collagen I prevented binding studies at a range of concentrations. A slow dissociation rate (Fig. 8). The limited solubility of the pN-collagen to PCPE was observed, and the shape of the curve obtained with the entire procollagen I molecule with a very similar sensorgram showed similar characteristics to the binding of pN-collagen to PCPE. Experimental curves are shown in white. Also shown (in black) are the best-fit curves obtained by global fitting using a simple bimolecular interaction model. B. plots of residuals corresponding to the differences between the experimental and best-fit curves.

Because the binding of the intact procollagen molecule to PCPE (K_D = 1 nM) appeared to be tighter than the binding of isolated C-propeptide trimers (K_D = 150–400 nM), it is likely that there might be additional sites for PCPE binding elsewhere in the procollagen molecule. To test this hypothesis, we measured the binding of pN-collagen I (i.e., procollagen lacking the C-propeptide region) to immobilized PCPE. Specific binding of pN-collagen to PCPE was observed, and the shape of the corresponding sensorgram showed similar characteristics to those obtained with the entire procollagen I molecule with a very slow dissociation rate (Fig. 8). The limited solubility of the pN-collagen I prevented binding studies at a range of concentrations. These data suggest that PCPE binds to sites in the procollagen molecule in addition to those in the C-propeptide region.

**DISCUSSION**

We report here that the C-propeptide trimers of procollagens I and III bind Ca^{2+}. Such binding has been demonstrated previously for the C-propeptide trimer of procollagen II, also known as chondrocalcin (48, 49), as well as for the collagen X molecule (46). These are in addition to the group of known Ca^{2+}-binding extracellular matrix proteins that includes SPARC/BM-40/osteonectin, fibrillin, and COMP, for which a number of different types of Ca^{2+} binding sites have been described (50, 51). Using PROSCAN (52), we found that the C-propeptide domains of the pro-c-1 chains of mammalian procollagens I, II, and III all contain a strongly conserved sequence (Cys-47 to Cys-73 following the BMP-1 cleavage site, numbered according to the pro-c-1(1) sequence), which shows 79% similarity to the Ca^{2+}-binding C-type lectin domain motif as found, for example, in MBL. Furthermore, within this sequence residues 59 to 71 are 74% similar to the Ca^{2+}-binding EF-hand domain motif as found in SPARC (53), although the invariant Glu or Asp at position 12 is lacking. This suggests that the Ca^{2+} binding site(s) may be localized to these sequences. In addition, the positions of ~50% of the acidic residues in the C-propeptide domains are strongly conserved, half of which are found in two acidic clusters in the C-terminal region (residues 180–246). Because relatively weak calcium binding can also be attributed to glutamic acid- or aspartic acid-rich sequences (50, 51), these also may represent potential Ca^{2+} binding sites.

Possible interaction sites for PCPE in the procollagen C-propeptide region can be proposed on the basis of recent small angle x-ray scattering studies of CPI (37). These indicate a structure consisting of three major lobes, each of which might correspond to the C-terminal region of each component chain containing the internal disulfide bonds, plus one minor lobe corresponding to a putative N-terminal junction region (residues 1–80) containing the interchain disulfide bonds. In view of our observation here that only one molecule of PCPE binds to the procollagen C-propeptide trimer, which is also supported by enzyme kinetic studies (1, 16), we speculate that the binding site is in this N-terminal junction region. Furthermore, because
interaction with PCPE did not prevent binding of mAb 48D34 to the 30 N-terminal residues of CPIII, this might further limit the possible binding site to residues 31 to 80, which contains all the interchain disulfide bonds (54) as well as the putative calcium binding site(s) (see above). Finally, the binding to the junction region is consistent with the observed lack of binding to PCPE following reduction and alkylation of CPIII, conditions that are likely to result in dissociation of the three polypeptide chains and/or changes in the three-dimensional structure.

The interaction studies described here on PCPE give insights into how it might stimulate the activity of PCP/CPIII. Such a mechanism would be possible if PCPE binds only to the liberated C-propeptide trimer (KD, 150–400 nM) and that this binding appears to be tighter than that to the isolated C-propeptide trimer (KD, 1–100 nM). Thus the “facilitating product release” hypothesis (Fig. 9A) seems unlikely.

The second hypothesis for PCPE action, originally proposed by Kessler and co-workers (16), is that PCPE binding to procollagen brings about a conformational change in the substrate, thereby facilitating cleavage by PCP (Fig. 9B). The observation here that PCPE binds to the C-propeptide domain as well as to additional sites in the procollagen molecule is consistent with such a mechanism, although the precise locations of the binding sites remain to be determined. It seems unlikely that PCPE binds to the N-propeptide domain in view of the length and rigidity of the procollagen molecule (PCP shows no enhancing activity on procollagen N-proteinase (32)). A more likely possibility is that PCPE binds within the mature collagenous region not far away from the PCP cleavage site. In this way, PCPE binding to sites within both the C-propeptide and collagenous regions would involve an additional contact interface that might lead to a conformational change in the cleavage site, thus facilitating PCP action (Fig. 9B). It should be noted that the data for procollagen binding to PCPE were equally well fitted to B and B.

Collagen brings about a conformational change in the substrate, regions would involve an additional contact interface that might lead to a conformational change in the cleavage site, thus facilitating PCP action (Fig. 9B). It should be noted that the data for procollagen binding to PCPE were equally well fitted to B and B.

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