Strong Cooperativity and Loose Geometry between CUB Domains Are the Basis for Procollagen C-Proteinase Enhancer Activity*S

Received for publication, July 17, 2009, and in revised form, September 11, 2009. Published, JBC Papers in Press, October 1, 2009, DOI 10.1074/jbc.M109.046128

Daniel Kronenberg‡§, Sandrine Vadon-Le Goff‡, Jean-Marie Bourhis‡, Bernard Font†, Denise Eichenberger‡, David J. S. Hulmes†, and Catherine Moali‡

From the ‡Institut de Biologie et Chimie des Protéines, CNRS/Université de Lyon UMR 5086, IFR128 Biosciences Gerland Lyon Sud, 7, Passage du Vercors, 69367 Lyon Cedex 7, France and the †Institute of Zoology, Cell and Matrix Biology, Johannes Gutenberg University, Johannes-von-Müller-Weg 6, 55128 Mainz, Germany

Procollagen C-proteinase enhancers (PCP-1 and -2) specifically activate bone morphogenetic protein-1 (BMP-1) and other members of the procollagen family during C-terminal processing of fibrillar collagen precursors. PCPEs consist of two CUB domains (CUB1 and CUB2) and one NTR domain separated by one short and one long linker. It was previously shown that PCPEs can strongly interact with procollagen molecules, but the exact mechanism by which they enhance BMP-1 activity remains largely unknown. Here, we used a series of deletion mutants of PCPE-1 and two chimeric constructs with repetitions of the same CUB domain to study the role of each domain and linker. Out of all the forms tested, only those containing both CUB1 and CUB2 were capable of enhancing BMP-1 activity and binding to a mini-procollagen substrate with nanomolar affinity. Both these properties were lost by individual CUB domains, which had dissociation constants at least three orders of magnitude higher. In addition, none of the constructs tested could inhibit PCPE activity, although CUB2/CUB2NTR was found to modulate BMP-1 activity through direct complex formation with the enzyme, resulting in a decreased rate of substrate processing. Finally, increasing the length of the short linker between CUB1 and CUB2 was without detrimental effect on both activity and substrate binding. These data support the conclusion that CUB1 and CUB2 bind to the procollagen substrate in a cooperative manner, involving the short linker that provides a flexible tether linking the two binding regions.

Tolloid proteinases have been shown to play important roles during embryogenesis and tissue remodeling. They control extracellular matrix synthesis as well as morphogenetic events such as dorso-ventral patterning, neural differentiation, and muscle growth (1). This control is achieved through proteolytic modifications of several matrix components (fibrillar and non-fibrillar procollagens, small leucine-rich proteoglycans, laminin 332, perlecan, and others), enzymes (lysyl oxidases) and growth factors or associated molecules (chordin, latent transforming growth factor-β-binding protein-1, growth differentiation factors 8 and 11, prolactin, and others). In mammals, the toolloid family includes bone morphogenetic protein-1 (BMP-1), mammalian toolloid, and mammalian toolloid like-1 and -2 (2). BMP-1 and mammalian toolloid are also known as “procollagen C-proteinases” (PCPs), because one of their key functions is to activate collagen fibrillogenesis through cleavage of the C-terminal propeptides in fibrillar procollagens (3). Collagen fibrils then provide a scaffold for further deposition of other matrix molecules.

Tolloid enzymes are assisted during collagen maturation by the procollagen C-proteinase enhancers-1 and -2 (PCP-1 and -2), which can increase toolloid activity on the major fibrillar procollagens by >10-fold (4, 5) while not affecting the cleavage of other known toolloid substrates (6). PCP-1s are rather small extracellular glycoproteins (~50 kDa) consisting of, from the N to the C terminus, two CUB domains and one NTR domain. These domains are separated by two linkers: one short linker (9 amino acids in human PCP-1) between the two CUB domains and one rather long linker (44 amino acids in human PCP-1) between the second CUB and the NTR domain. CUB domains were originally found in proteins from the complement system, in the sea urchin protein Uegf and in BMP-1, whereas the NTR domain shares homology with the C-terminal domain of netrins whose primary role is in axonal guidance (7).

The mechanism by which PCPEs increase toolloid activity in such an efficient and specific manner is only partially understood. One major determinant of this efficiency seems to be the direct and strong interaction of PCP-1s with fibrillar procollagen substrates (4, 8), whereas interaction of PCP-1s with toolloid proteinases is possible but probably much weaker (9). Interestingly, the CUB domain region alone is sufficient to promote enhancement (4, 10). We have shown that the triple helix of procollagens is not required for toolloid stimulation (6) and have identified several residues in CUB1 that seem to play a major role in the PCP-1-procollagen interaction (11). Of note, when the calcium-binding site in CUB1 is disrupted, interaction with the C-terminal part of procollagen III is completely abolished.

*This work was supported by the Région Rhône-Alpes, the European Commission (contract NMP2-CT-2003-504017), the Agence Nationale de la Recherche, the CNRS, and the Université Claude Bernard Lyon 1.
²The abbreviations used are: BMP-1, bone morphogenetic protein-1; PCP, procollagen C-proteinase; PCPE, PCP enhancer; ELISA, enzyme-linked immunosorbent assay.
locating one possible interaction site on loops 5, 7, and 9 of CUB1. One attractive hypothesis is that PCPEs actually bind to both sides of the tolloid cleavage site (located between the C-telopeptide and the C-propeptide), thereby inducing a conformational change in the procollagen molecule and facilitating procollagen C-proteinase action (8).

Here, using a series of novel constructs derived from PCPE-1 and resulting from the deletion of one or more domains or from domain-swapping, we show that although both CUBs are required for enhancement, they bind very weakly to the substrate as individual domains. Also, we show that exchanging one CUB domain for another CUB domain results in complete loss of enhancing activity and can even lead to tolloid inhibition. In contrast, increasing the length of the short linker region between CUB1 and CUB2 has no significant effect. Altogether, our present data give further insight into previously proposed mechanisms and raise new possibilities to explain PCPE action.

EXPERIMENTAL PROCEDURES

Molecular Biology—New DNA constructs used in this study are CUB1NTR, CUB2NTR, CUB1CUB1NTR, CUB2CUB2NTR, and PCPE+4 (Fig. 1A). CUB1NTR and CUB2NTR were produced with the QuikChange XL site-directed mutagenesis kit from Stratagene using the cDNA of human PCPE-1 inserted into the pCEP4 vector (Invitrogen), in-frame with an 8-histidine C-terminal tag (known as PCPEhis) (11). 5’-Phosphorylated primers were designed to delete residues 150–275 to obtain CUB1NTR (numbering according to human full-length PCPE-1, including the signal peptide) and residues 26–149 to obtain CUB2NTR. CUB2CUB2NTR corresponds to the insertion of residues 162–273 (CUB2) in place of residues 40–149 (CUB1). This was obtained after ligation of the 5’-phosphorylated PCR product corresponding to residues 162–273 directly in an opened PCR product resulting from deletion of the region corresponding to residues 40–149 in PCPEhis inserted in pCR-BluntII-TOPO (Invitrogen). The resulting construct was subcloned in pCEP4 using the KpnI and BamHI sites. The same procedure applied to CUB1CUB1NTR failed to yield the desired product so, instead, insertion of residues 40–149 (CUB1) in place of residues 162–273 (CUB2) was performed by addition of a SacI restriction site on the 5’-end of the fragment coding for CUB1 and a NotI site on its 3’-end. Ligation of this product in the PCPEhis + pCR-BluntII-TOPO construct, modified to accept the SacI-NotI fragment, and further subcloning in pCEP4 yielded the construct called CUB1CUB1NTR. This construct contains four additional residues (ELQF), not present in PCPE-1, at the end of the linker between the CUB1 domains. Finally, the same four additional residues were inserted after F158 in PCPEhis + pCR-BluntII-TOPO using primers overlapping in the region to be inserted with 5’-phosphorylated and 6-base floating ends. The PCR product was again ligated, transformed, and subcloned into pCEP4, resulting in the construct called PCPE+4.

Protein Production, Purification, and Characterization—Human forms of mini-procollagen III, PCPE, PCPEhis, and BMP-1-FLAG were produced in 293-EBNA cells as previously described (6, 11). Similarly, the pCEP4 constructs described in the previous section were transfected in 293-EBNA cells using Lipofectamine as transfection agent. After 24 h, hygromycin B (300 μg/ml) was added to the medium, and selection was maintained for the duration of cell amplification. Protein production was performed in serum-free conditions for up to 3 weeks, and medium was collected every 2 days. Cell supernatants were clarified by centrifugation and batch purified with nickel-nitritriacetic acid-agarose (Qiagen, 20 ml/liter) for 3 h at 4 °C (11). The gel was then loaded into a column, and His-tagged proteins were eluted with 250 mM imidazole in 50 mM NaH2PO4, pH 8, 0.3 M NaCl. Imidazole was removed either by dialysis or by diluting protein fractions 3-fold followed by loading onto a 5-ml heparin HiTrap column (Amersham Biosciences). CUB1CUB2, CUB1CUB2+4, CUB1, CUB2, and NTR were obtained by limited proteolysis of, respectively, PCPEhis, PCPE+4, CUB1NTR, and CUB2NTR on agarose-immobilized trypsin (Sigma, ~1 mg of protein was incubated with 500 μl of slurry for 15 min at room temperature in a total volume of 3 ml of 20 mM HEPES, pH 7.4, 0.15 M NaCl, 5 mM CaCl2). CUB containing fragments, found in the flow-through and wash fractions, were separated from the NTR domain on a heparin HiTrap column.

N-Glycosidase F (Roche Applied Science) was used according to the manufacturer’s instructions with native CUB1. Mass spectrometry and N-terminal sequencing were performed at the facility for protein microsequencing (IFR128, Gerland). Matrix-assisted laser desorption ionization time-of-flight mass spectra were recorded on a Voyager DE-PRO (Applied Biosystems) instrument in the 2.5- to 25-kDa mass range using linear mode and external calibration. The matrix was a sinapinic acid solution (Laser BioLabs, 1 mg/100 μl in 50% CH3CN/5% H2O/0.1% trifluoroacetic acid).

CD Spectroscopy—To evaluate the folding of the PCPE-1 fragments, far UV (195–260 nm) CD measurements were carried out using thermostatted 0.2-mm path length quartz cells in an Applied Photometrics Chirascan instrument, calibrated with aqueous d-10-camphorsulfonic acid. Proteins (~0.5 mg/ml) were analyzed at 25 °C in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, in the presence and absence of 10 mM CaCl2. Spectra were measured with a wavelength increment of 0.5 nm, integration time of 1–6 s, and bandpass of 1 nm. Protein concentrations were determined by absorbance at 280 nm using the absorbance calculated from the amino acid sequence. To calculate simulated curves for full-length PCPEhis, the observed mean residue ellipticities for the CUB1 and CUB2NTR fragments were combined after weighting according to the number of residues in each protein.

Labeling of Mini-procollagen III—Mini-procollagen III was covalently labeled with the fluorescent dye DyLight633-NHS ester (ThermoScientific) according to the manufacturer’s recommendations. Briefly, 100–500 μl of mini-procollagen III at 1 mg/ml in 50 mM borate buffer, pH 8.5, was transferred to a 50-μg vial of dye, and the mixture was incubated at room temperature for 1 h. Excess reagent was removed using dye-removal columns (ThermoScientific), and the labeled protein was aliquoted and stored at ~80 °C in black tubes. The number of fluorescent labels per molecule of mini-procollagen III was calculated from the emission maximum (630 nm) of the dye fluorescence spectrum after coupling, according
**PCPE** Mechanism of Action

K. J. McCauley, Y. Li, D. W. Gerber, T. S. Conaway, C. J. L. Conaway

J. Biol. Chem. 284: 33439

NOVEMBER 27, 2009-VOLUME 284 • NUMBER 48

**Production and Characterization of PCPE Constructs**—Since the discovery of PCPE-1 by Adar and co-workers, it is known that proteolytic fragments of PCPE-1, encompassing the CUB1 and CUB2 domains (Fig. 1A), retain an enhancing activity that seems similar to the activity of the full-length molecule (4, 17). In the present study, we produced and purified nine recombinant proteins derived from human PCPE-1 or its His-tagged version to precisely analyze the role of each domain and determine which combinations of domains result in enhancing activity. These constructs are described in Fig. 1A and include: (i) shorter proteins, for which one CUB domain has been deleted (CUB1NTR and CUB2NTR), (ii) chimera proteins with contiguous repetitions of the same domain (CUB1CUB1NTR and CUB2CUB2NTR), and (iii) a slightly longer protein where four amino acids were inserted just before the beginning of the CUB2 domain yielding a longer linker region between CUB1 and CUB2 (PCPE+4). The inserted sequence was ELQF to resemble a sequence already found in the linker (Fig. 1A) and to serve as a control for CUB1CUB1NTR, in which this sequence was also inserted for cloning purposes.

Attempts to produce CUB1, CUB2, or CUB1CUB2 in 293-EBNA cells yielded very low amounts of protein compared with the amounts obtained with other constructs, suggesting that the NTR domain was required for proper folding or secretion. To circumvent this problem, another approach was chosen that consisted of submitting PCPEhis, PCPE+4, CUB1NTR, and CUB2NTR to limited proteolysis, then separating fragments on...
PCPE Mechanism of Action

Heparin-Sepharose. Complete cleavage of the parent proteins was obtained after incubation with trypsin-agarose for 15 min (Fig. 1B, lanes 1 and 2). Cleavage occurred mainly in the long linker preceding the NTR domain but at several sites yielding three major fragments on a SDS-PAGE 13% gel (Fig. 1B, lanes 3 and 4). The two fragments retained on heparin-Sepharose (lane 4) correspond to the NTR domain starting in the linker at serine 300 or threonine 306 (numbering for full-length PCPE-1, including the signal peptide), as determined by N-terminal sequencing. A longer fragment resulting from incomplete cleavage could also be detected in some preparations, starting at valine 294.

The fragments that were not retained on the heparin column were more homogeneous and corresponded to CUB1, CUB2, to the intact protein. Furthermore, the spectrum of the intact CUB2NTR fragment was unchanged after limited proteolysis with immobilized trypsin (supplemental Fig. S2B), showing that separation of the CUB2 and NTR domains had no effect on their folding. The presence or absence of calcium also had no effect on the observed spectra.

Only PCPE-1, CUB1CUB2, PCPE+4, and CUB1CUB2+4 Have Enhancing Activity—We first checked if the recombinant CUB1CUB2 protein was capable of enhancing BMP-1 activity, as would be expected from previous reports using proteolytic fragments of mouse PCPE-1 (4, 10). When a model substrate composed of a c-Myc tag, a short triple helix including the last 33 triplets, the C-telopeptide, and the C-propeptide of procollagen III was used as a substrate

FIGURE 1. PCPE constructs used in this study. A, schematic representation of the constructs derived from human PCPE-1 (PCPE) used in the present study. Numbers indicate first amino acid for (from left to right) N-terminal extension, CUB1, short linker, CUB2, long linker, NTR, and C-terminal extension. Inserted sequences are those of the short linker in PCPE-1 and the sequence of the insertion present in PCPE+4, CUB1CUB1NTR, and CUB1CUB2+4. His = His tag. B, SDS-PAGE showing the products of PCPEhis (lane 1) obtained after trypsinolysis (lane 2) and separation on heparin-Sepharose (lane 3, flow-through containing CUB1CUB2; lane 4, elution fraction containing NTR). The gel was run in reducing conditions (13% acrylamide) and stained with Coomassie Blue.

FIGURE 2. Enhancing effects of PCPE constructs on BMP-1 activity. Samples were analyzed by SDS-PAGE on 4–20% gradient gels stained with Coomassie Blue under non-reducing conditions. The molar ratio mini-procollagen III/construct was 1/1. Asterisks indicate lanes where an enhancing effect of the tested construct on mini-procollagen III processing by BMP-1 can be seen. Minili, mini-procollagen III; CPIII, C-propeptide III; and N-ter, N-terminal cleavage product.
(mini-procollagen III (6)), we observed that CUB1-CUB2 induced a total conversion of the substrate by BMP-1, similarly to PCPE and PCPEhis (Fig. 2). In this assay, the molar ratio of enhancer over substrate was 1:1. In contrast, when mini-procollagen III was incubated with BMP-1 in the presence of other constructs, including CUB1, CUB2, NTR, CUB1NTR, CUB2NTR, CUB1CUB1NTR, and CUB2CUB2NTR (Fig. 2), no visible enhancing effect was observed. Importantly, when an equimolar mixture of CUB1 and CUB2NTR was added, the PCPE-like activity could not be reconstituted indicating that the short linker between the two CUB domains plays a crucial role. However, modifying this region by insertion of four additional amino acids, as in PCPE/H11001 and CUB1CUB2/H11001, did not lead to significant changes in the enhancing activity of PCPEhis. This result also shows that this additional sequence, which was also introduced in CUB1CUB1NTR for cloning purposes, is not responsible for the loss of enhancing activity observed with the latter construct. Altogether, these experiments clearly indicate that: (i) the two contiguous CUB domains are required for the stimulation of BMP-1, (ii) these CUB domains cannot be exchanged, and (iii) even though the short linker between the two CUB domains is absolutely required, some flexibility is still permitted within this region.

**PCPE-1 Domains Are Inefficient Competitors of PCPE-1**—For a better understanding of why most of the PCPE constructs lost enhancing activity, we then searched to determine how their interactions with the substrate were modified. The first experiment was a competition assay where CUB-containing constructs were assessed for their ability to inhibit enhancement by PCPE-1. In all cases, a 6.6 molar excess of construct compared with PCPEhis was used. In these conditions, CUB2CUB2NTR was the only protein for which a significant reduction in the amount of C-propeptide formed could be observed (Fig. 3). In another experiment, BMP-1 alone was assayed in the presence of mini-procollagen III and CUB2CUB2NTR, and we observed that CUB2CUB2NTR also decreased substrate processing in this case (data not shown).

This result suggested that CUB2CUB2NTR inhibited BMP-1 through a direct effect rather than through competition with PCPE. Unexpectedly, our preliminary experiments also seemed to indicate that, although partial inhibition could be detected in the presence of 10 nM CUB2CUB2NTR, BMP-1 retained some activity even at very high concentrations of the construct (up to 2 μM).

To better quantify BMP-1 activity in the presence and absence of CUB2CUB2NTR, we decided to develop a new quantitative and non-radioactive assay. We thus labeled recombinant mini-procollagen III with the fluorescent dye DyLight633 from ThermoScientific, a fast and easy procedure that targets free amines (N terminus and lysines; see “Experimental Procedures” for details). When 50 μg of labeling reagent was mixed with a 1 mg/ml solution of mini-procollagen III, we calculated that between 1.3 and 2 molecules of label had reacted with mini-procollagen III. This labeled substrate could still be cleaved by BMP-1, and this cleavage was enhanced by PCPE-1, as shown in Fig. 4A. Both cleavage products could be separated on a gel and
PCPE Mechanism of Action

A. PCPE Response (RU) over Time (s)

B. PCPEhis Response (RU) over Time (s)

C. CUB1CUB2 Response (RU) over Time (s)

D. CUB1CUB2+4 Response (RU) over Time (s)

E. CUB1 Response (RU) over Time (s)

F. CUB2 Response (RU) over Time (s)

G. NTR Response (RU) over Time (s)

H. OD 405 (normalized) vs. Construct (nM)
quantified on a gel scanner equipped with a red laser. Of note, the N-terminal product, including the triple helix and the C-telopeptide, gave a weaker signal than the C-propeptide (Fig. 4A), in agreement with a smaller number of potential reactive sites (5 compared with 19). The plot of measured mini-procollagen III fluorescence intensity versus the amount of protein was found to be linear up to 500 ng of protein loaded per well (data not shown).

Using increasing concentrations of CUB2CUB2NTR (10–500 nM) with fixed concentrations of fluorescent mini-procollagen III and BMP-1, we confirmed that CUB2CUB2NTR was a BMP-1 inhibitor, but that maximum inhibition was only 40% even when enzyme was saturated with inhibitor (above 100 nM) (Fig. 4B). Such a partial inhibition is usually referred to as “hyperbolic inhibition” because apparent Michaelis parameters depend on inhibitor concentration both in the numerator and denominator (18). It implies that the enzyme-inhibitor complex is still active, unlike “linear inhibition” modes where formation of the enzyme-inhibitor complex is a dead-end but has a slower catalytic constant than the free enzyme (19). Interestingly, when a fluorogenic peptide was used as a substrate (20), a similar behavior was obtained with CUB2CUB2NTR, whereas PCPEhis seemed to have no effect on the peptide (Fig. 4B). As a consequence, CUB2CUB2NTR appears to be a general modulator of BMP-1 activity. Although a thorough kinetic analysis of BMP-1 inhibition by CUB2CUB2NTR was beyond the scope of this report, we did determine the dissociation constant of the BMP-1-CUB2CUB2NTR complex by ELISA and found it to be 56 ± 17 nM (data not shown).

**PCPE Mechanism of Action**

In the last part of this study, we determined dissociation constants for all constructs and mini-procollagen III. The first technique used was surface plasmon resonance on a Biacore T100 apparatus. Mini-procollagen III was covalently coupled to a CM5 sensor chip, and the various PCPE constructs were injected in a buffer containing 5 mM calcium, the concentration used in the activity assays. All constructs were found to interact with mini-procollagen III, but the concentrations required varied over more than four orders of magnitude. When we compared PCPE and PCPEhis (Fig. 5, A and B), it also appeared that the His tag present on several of our constructs could impair the quality of the recorded sensorgrams during the association phase, a phenomenon that was not observed previously on a Biacore 3000 in the absence of calcium (11). This effect was diminished when decreased flow rates and concentrations were used or when measurements were made in the absence of calcium, but it precluded reliable kinetic analysis of the binding curves. For this reason and because kinetic association and dissociation constants were not required for this study, we decided to use ELISA in parallel to assess dissociation constants of His-tagged constructs.

Comparison of the two techniques in the case of PCPE and CUB1CUB2 gave values that fell in the same range (Table 1). However, the Biacore curves are more informative and can reveal more subtle effects such as the presence of two binding sites. In the present case, curves for PCPE and CUB1CUB2 (Fig. 5, A and C) were best fitted with the heterogeneous ligand model assuming that there are two possible binding sites on mini-procollagen III. Two constants were thus derived, one around 0.4 nM and one around 8 nM (Table 1). The constants for PCPE were significantly lower than those previously determined for PCPEhis and mini-procollagen III (1.8 and 48 nM (11)), and this is explained by the addition of calcium in the present experiments, which significantly strengthens the interactions. Importantly, we also conclude from this that CUB1CUB2 binds to mini-procollagen III with dissociation constants similar to those for PCPE.

To further assess the consequences of lengthening the linker between the two CUB domains, we also used CUB1CUB2+4, prepared from PCPE+4 by limited proteolysis, for Biacore

**TABLE 1**

**Dissociation constants of PCPE constructs with mini-procollagen III determined by Biacore or ELISA**

Means of three independent experiments (with different surfaces for Biacore) ± S.D. For the Biacore studies of PCPE and CUB1CUB2, the best fits were obtained with the “heterogeneous ligand” model (values in parentheses show the mean contribution of each interaction site expressed as a percentage of the total signal); for CUB1CUB2+4 and NTR, the best model was “1:1 binding” and for CUB1 and CUB2, the $K_d$ values were obtained from the steady-state responses plotted against CUB concentrations. $\chi^2$ values were all <3.6. Representative curves and fits are shown in Fig. 5.

|        | PCPE | CUB1CUB2 | CUB1CUB2+4 | CUB1 | CUB2 | NTR |
|--------|------|---------|------------|------|------|-----|
| Biacore | 0.33 ± 0.10 (47%) | 0.54 ± 0.18 (61%) | 5.6 ± 0.7 | 7800 ± 4300 | >10,000 | 770 ± 480 |
| ELISA  | 1.5 ± 0.5 | 2.2 ± 0.6 | ND* | ND | ND | ND |
| PCPEhis | 2.4 (53%) | 0.10 (47%) | 3.4 (39%) | 0.6 | ND | ND |
| CUB1NTR | 6.4 | 26 ± 9 | 220 ± 90 | 37 ± 18 | 790 ± 280 |

* ND, not done; NF, curves could not be fitted with available models.
PCPE Mechanism of Action

analysis (Fig. 5D). In these conditions, we found a unique $K_D$ of 5.6 ± 0.7 nm, which is comparable to the higher of the two $K_D$ values found for CUB1CUB2 and PCPE. The addition of four residues in CUB1CUB2 seemed to prevent binding of the protein to the higher affinity site but did not modify the lower affinity site. Because PCPE+4 and CUB1CUB2+4 were found to be as active as PCPE and CUB1CUB2 in the conditions used, this would suggest that the high affinity site does not play a major role in the enhancing activity of the proteins studied.

Unexpectedly, individual CUB1 and CUB2 domains were found to bind to mini-procollagen III with very high dissociation constants (7.8 μM for CUB1 and even higher for CUB2 (Fig. 5, E and F, and Table 1)). Both the association and dissociation kinetics were actually very fast for the two constructs, and because they were out of the Biacore specifications, we used a steady-state analysis to determine $K_D$ values. Even in this case, saturation was difficult to reach in a reasonable concentration range, and it was only for CUB1 that a $K_D$ could be derived (inset in Fig. 5E). Noteworthy, using less detergent (0.005% instead of 0.05% P20) in the running buffer of the Biacore experiments, a significant improvement of the signal obtained with CUB2 was observed (reaching 30–50 resonance units at 10 μM for 700 resonance units of immobilized mini-procollagen III). This shows that CUB2 can also bind mini-procollagen III but much more weakly than other constructs. The fact that the dissociation constants for CUB1 and CUB2 were at least $10^4$-fold higher than the values found for CUB1CUB2 is probably the most striking of all and clearly suggests that CUB1 and CUB2 bind to the procollagen substrate in a cooperative manner. This confirms that CUB1 and CUB2 cannot be efficient competitors of PCPE, as described in the previous section. Interestingly, the NTR domain which, from previous results, is not expected to play a role in the mechanism of action of PCPE, interacted with mini-procollagen III with a lower $K_D$ (0.77 μM) than the individual CUB domains, which are essential for activity. From the ELISA experiments, we can also deduce that CUBNTR constructs show significant improvements in their affinities for mini-procollagen III compared with single domain constructs (Fig. 5H and Table 1). The CUB1NTR construct, which brings together two normally distant domains, had a surprisingly high affinity for the substrate with a $K_D$ of 26 ± 9 nm. Addition of a third domain to the CUBNTR constructs had only a weak effect on the interaction with mini-procollagen III and even seemed detrimental in the case of CUB2CUB2NTR compared with CUB2NTR. Moreover, the $K_D$ of CUB2CUB2NTR for mini-procollagen III was much higher than the concentration required for maximum inhibition of BMP-1 (see above). This gives strength to the hypothesis that this construct hinders cleavage of substrate by BMP-1 through a direct interaction with the enzyme rather than through an interaction with the substrate, as was first expected.

DISCUSSION

Extracellular proteases are known to be regulated by a complex “arsenal” of endogenous inhibitors (e.g. serpins for serines proteases, cystatins for cathepsins, tissue inhibitors of metalloproteinases for extracellular metalloproteases). Mammalian tolloid proteinases are rather unique among extracellular pro-
immobilized antigen: even though the affinities for individual antigen binding sites are identical, binding to the second site is faster once the first binding site is occupied. Similarly, both binding sites must release at the same time in order for the antibody molecule to dissociate. The effect is to increase the apparent strength of the binding. By analogy, the role of the short linker in PCPEs would be similar to that of the Fc region in an antibody molecule, to provide a flexible tether linking the two binding regions. In contrast to the antibody-antigen interaction, however, both interacting regions in PCPEs (the CUB domains) are binding to different sites on the same target molecule (procollagen), and the individual affinities are slightly different.

Other mechanisms can contribute to the strong cooperativity between the two CUB domains. For example, CUB1 could bind and induce a conformational change in the procollagen molecule that would facilitate the binding of CUB2. Alternatively, interaction of CUB1 with procollagen could create a completely new interaction surface for CUB2, with amino acids from both CUB1 and procollagen contributing. From the Bia-core curves, we saw that dissociation of CUB1 from mini-procollagen III was very fast (too fast to yield reliable kinetic parameters), and even when injection times were increased up to 10 min, there was no evidence of a conformational change that would decrease the dissociation rate of the complex (data not shown). Moreover, when taken alone, these mechanisms would probably imply that a mixture of CUB1 and CUB2NTR should activate procollagen processing and this is not the case. Altogether, the avidity hypothesis seems the most attractive, but contributions from the two other mechanisms cannot be excluded.

To further assess the flexibility of the linker, we calculated thirteen independent models from the previously obtained small angle X-ray scattering data for PCPE-1 (15) using BUNCH software (which was not available at the time of the earlier study). This new program allows the simultaneous determination of the optimal positions and orientations of each domain (CUB1, CUB2, and NTR) and of the probable conformations of the flexible linkers. From these calculations, it is clear that the angle between CUB1 and CUB2 can vary significantly, from approximately −90° to +90° (Fig. 6), with all configurations fitting the previously determined curve. This is also shown by the measure of the root mean square deviation between each pair of CUB2 domains, which revealed a large dispersion from 10 to 30 Å. Thus, the CUB1 and CUB2 domains seem to be able to explore several relative orientations, and this leads us to conclude that, in solution and in the absence of a binding partner, PCPE-1 shows an inherent flexibility between its two CUB domains.

CUBNTR constructs also show some degree of cooperativity as they bind more strongly to mini-procollagen III than individual domains. Although this could be expected for CUB2NTR, which is a “natural” domain association, it was more surprising for CUB1NTR, which brings together two domains that are not contiguous in PCPE-1. This unnatural fusion does not seem to preclude interaction of each domain with its normal binding site and could even facilitate these interactions for the reasons described above. It should be noted here that the CUB1NTR linker is the same as that normally found between CUB2 and NTR in PCPE-1, a 44-residue long sequence that is predicted to be intrinsically disordered and very flexible.3 No further gain in affinity was observed in CUBCUBNTR constructs (containing identical CUB domains) compared with CUBNTR constructs. With CUB2CUB2NTR, even a decrease in affinity was observed, and this construct bound to mini-procollagen III with a dissociation constant similar to that of the NTR domain alone. However, the CUB2CUB2NTR construct, but not the NTR domain alone, was shown to modulate BMP-1 activity with a completely new mechanism. CUB2CUB2NTR does not bind mini-procollagen III very strongly but binds and inhibits BMP-1 in the same range of concentrations, suggesting that its inhibitory effect was mediated by a direct interaction with the enzyme. Despite the fact that cleavage of a small fluorescent peptide was also inhibited by CUB2CUB2NTR, it is more likely that this protein does not bind in the catalytic pocket of BMP-1, because processing remains possible at high CUB2CUB2NTR concentrations. In conclusion, we can

3 M. Bekhouche, D. Kronenberg, S. Vadon-Le Goff, C. Bijakowski, N. H. Lim, B. Font, A. Colige, H. Nagase, G. Murphy, D. J. S. Hulmes, and C. Moali, manuscript in preparation.
assume that CUB2CUB2NTR binds in close proximity to the enzyme active site, thereby decreasing the reaction rate.

One negative aspect of the fact that CUB domains and CUBNTR proteins are not efficient PCPE antagonists is that these constructs cannot be used as therapeutic tools to counteract PCPE action. Because of their specificity in enhancing procollagen processing, in contrast to tolloid proteinases, which have a wide range of substrates, PCPEs would appear to be attractive targets in the prevention of fibrosis and hypertrophic scarring. PCPE antagonists could serve to slow down collagen deposition during the repair process, thereby improving the quality of the remodeled tissue. Because single domain PCPE constructs cannot be used, other therapeutic strategies will have to be investigated. Interestingly, a new approach using chimeric proteins derived from PCPEs is suggested here with CUB2CUB2NTR.

Acknowledgments—We are grateful to Dominique Mazzocut for N-terminal sequencing, Michel Becchi and Isabelle Zanella-Cléon for mass spectrometry analysis, Yannick Tauran and Annie Chaboud for excellent Biacore maintenance, and Eve Pécheur for technical assistance during the use of the TECAN fluorometer.

REFERENCES
1. Ge, G., and Greenspan, D. S. (2006) Birth Defects Res. C. Embryo. Today 78, 47–68
2. Hopkins, D. R., Keles, S., and Greenspan, D. S. (2007) Matrix Biol. 26, 508–523
3. Hulmes, D. J. (2008) in Collagen—Structure and Mechanics (Fratzl, P., ed) pp. 15–47, Springer, New York
4. Adar, R., Kessler, E., and Goldberg, B. (1986) Coll. Relat. Res. 6, 267–277
5. Steiglitz, B. M., Keene, D. R., and Greenspan, D. S. (2002) J. Biol. Chem. 277, 49820–49830
6. Moali, C., Font, B., Ruggiero, F., Eichenberger, D., Rousselle, P., François, V., Oldberg, A., Bruckner-Tuderman, L., and Hulmes, D. J. (2005) J. Biol. Chem. 280, 24188–24194
7. Bradford, D., Cole, S. J., and Cooper, H. M. (2009) Int. J. Biochem. Cell Biol. 41, 487–493
8. Ricard-Blum, S., Bernocco, S., Font, B., Moali, C., Eichenberger, D., Farnjel, J., Burchardt, E. R., van der Rest, M., Kessler, E., and Hulmes, D. J. (2002) J. Biol. Chem. 277, 33864–33869
9. Ge, G., Zhang, Y., Steiglitz, B. M., and Greenspan, D. S. (2006) J. Biol. Chem. 281, 10786–10798
10. Hulmes, D. J., Moald, A. P., and Kessler, E. (1997) Matrix Biol. 16, 41–45
11. Blanc, G., Font, B., Eichenberger, D., Moreau, C., Ricard-Blum, S., Hulmes, D. J., and Moali, C. (2007) J. Biol. Chem. 282, 16924–16933
12. Appleton, B. A., Wu, P., Maloney, J., Yin, J., Liang, W. C., Stawicki, S., Mortara, K., Bowman, K. K., Elliott, J. M., Desmarais, W., Bazan, J. F., Bagri, A., Tessier-Lavigne, M., Koch, A. W., Wu, Y., Watts, R. J., and Wiesmann, C. (2007) EMBO J. 26, 4902–4912
13. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
14. Liepinsh, E., Banyai, L., Pintacuda, G., Trexler, M., Pathy, L., and Otting, G. (2003) J. Biol. Chem. 278, 25982–25989
15. Bernocco, S., Steiglitz, B. M., Svergun, D. I., Petoukhov, M. V., Ruggiero, F., Ricard-Blum, S., Ebel, C., Geourjon, C., Deleage, G., Font, B., Eichenberger, D., Greenspan, D. S., and Hulmes, D. J. (2003) J. Biol. Chem. 278, 7199–7205
16. Kleywegt, G. J. (1996) Acta Crystallogr. D. Biol. Crystallogr. 52, 842–857
17. Kessler, E., and Adar, R. (1989) Eur. J. Biochem. 186, 115–121
18. Baici, A. (1981) Eur. J. Biochem. 119, 9–14
19. Cornish-Bowden, A. (2004) Fundamentals of Enzyme Kinetics, 3rd Ed., Portland Press, London
20. Lee, H. X., Ambrosio, A. L., Reversade, B., and De Robertis, E. M. (2006) Cell 124, 147–159
21. Moali, C., and Hulmes, D. J. (2009) Eur. J. Dermatol. 19, in press
22. Scott, I. C., Blitz, I. L., Pappano, W. N., Maas, S. A., Cho, K. W., and Greenspan, D. S. (2001) Nature 410, 475–478
23. Inomata, H., Haraguchi, T., and Sasai, Y. (2008) Cell 134, 854–865
24. Kobayashi, K., Luo, M., Zhang, Y., Wilkes, D. C., Ge, G., Grieskamp, T., Yamada, C., Liu, T. C., Huang, G., Basson, C. T., Kispert, A., Greenspan, D. S., and Sato, T. N. (2009) Nat. Cell Biol. 11, 46–55