Post-translational Proteolytic Processing of Procollagen C-terminal Proteinase Enhancer Releases a Metalloproteinase Inhibitor

(Received for publication, August 26, 1999, and in revised form, October 28, 1999)

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Activity of matrix metalloproteinases (MMPs) is regulated by a family of proteins called tissue inhibitors of metalloproteinases (TIMP). Four TIMPs have been cloned, and their molecular weights range from 29,000 to 20,000. By reverse zymography, we have observed a metalloproteinase inhibitor with an apparent molecular weight of 16,500 from medium conditioned by human brain tumor cells. Antibodies directed against TIMPs failed to react with the 16,500 molecular weight inhibitor, indicating that it was not a truncated form of a known TIMP. The inhibitor was isolated from conditioned medium using affinity and ion exchange chromatography. N-terminal sequences of the inhibitor matched amino acid sequences within the C-terminal domain of a protein known as procollagen C-terminal proteinase enhancer (PCPE). Thus, the inhibitor was named CT-PCPE. Comparison of the N-terminal domain of TIMP with CT-PCPE revealed that both contained six cysteine residues. As in the case of TIMP, reduction and alkylation abolished the inhibitory activity of CT-PCPE. Purified CT-PCPE inhibited MMP-2 with an IC50 value much greater than that of TIMP-2. This implies that MMPs may not be the physiologic targets for CT-PCPE inhibition. However, these results suggest that CT-PCPE may constitute a new class of metalloproteinase inhibitor.

Metzincins comprise a superfamily of zinc-dependent endoproteinases that include astacins, ADAM (a disintegrin and a metalloproteinase), and matrix metalloproteinases (MMP)1 (1). In many instances components of the extracellular matrix can serve as substrates for these metalloproteinases. Members of the metzincin family continue to be identified, and new functions are being recognized for enzymes previously described. Enzymes are classified into different metzincin families based on structural features of their catalytic domain. All metzincins are inhibited by chemicals that chelate the zinc ion (1). Until recently, protein inhibitors had been characterized for only the MMP family. Endogenous MMP activity is mainly regulated by a family of proteins called tissue inhibitors of metalloproteinases (TIMP) (2).

Four members of the TIMP family have been identified and are designated TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Molecular weights of these inhibitors range from 20,000 to 29,000 (3). Although the primary amino acid sequence identity between the TIMPs is low (3, 4), their tertiary structure is thought to be remarkably similar because of the conserved positions of 12 cysteine residues. These 12 cysteine residues form six disulfide bonds, and correct pairing of these residues is critical for TIMP inhibitory function (5–7). Although TIMP generally have a broad specificity with regard to MMP inhibition, TIMP-1 is often found associated with active and latent forms of MMP-9 (8), and TIMP-2 is often found associated with active and latent forms of MMP-2 (9–11). TIMP-3 is found associated with the extracellular matrix, and TIMP-4 may be expressed in a tissue-specific manner (2). More recently, the functions of TIMP have been broadened from inhibitors of only MMPs. TIMP-3 has been shown to inhibit TACE (tumor necrosis factor-a converting enzyme), a member of ADAM (12). However, the Kd derived for TIMP-3 inhibition of TACE was much larger than that derived for inhibition of MMP-2. Additionally, TIMP-1 has been shown to inhibit aggrecanase, a recently described ADAM 13, 14).

Because of their role in extracellular matrix remodeling, MMPs have been widely studied in tumor cell invasion and metastasis. Expression of both MMP and TIMP has been shown to increase in some tumor cell lines. Several studies have shown that increase in the expression of TIMP can decrease the invasiveness of some tumor cell lines. For example, transfection of TIMP-1 cDNA into astrocytoma cells diminishes their invasive potential (15, 16). Observations such as these suggest that modulation of metalloproteinase activity may be a significant control point for regulation of tumor cell invasion. Consequently, the presence of TIMP or other metalloproteinase inhibitors may be beneficial in controlling tumor cell invasion.

Previously, during the course of investigating TIMP activity in cell lines derived from human brain tumors, our laboratory observed at least four metalloproteinase inhibitors present in the conditioned medium. Their molecular weights ranged from 29,000 to 16,500. Based on their molecular sizes, the inhibitors were most likely TIMP-1, at a molecular weight of 29,000, TIMP-3 and/or TIMP-4 at a molecular weight of 23,000, and TIMP-2 at a molecular weight of 20,000. However, the smallest inhibitor, migrating at an apparent molecular weight of 16,500, did not correspond to the molecular size of any known TIMP, and it was not detected in normal cells derived from brain (17).
These observations suggested that the smallest inhibitor may be specific to transformed cells arising in human brain tumors. In the present study, the 16,500 molecular weight inhibitor has been isolated, identified, and partially characterized. Results of this study show that the 16,500 molecular weight inhibitor is not a new member of the TIMP family, nor is it a truncated form of a previously identified TIMP. Instead our results indicate that the novel inhibitor is the C-terminal fragment of procollagen C-terminal proteinase enhancer (PCPE).

**EXPERIMENTAL PROCEDURES**

**Isolation of CT-PCPE—**Once confluent, U343-MGA or H4 cell lines were grown under serum-free conditions with 0.2% lactalbumin hydrolysate added to Dulbecco's modified Eagle's medium in roller bottles. U343-MGA, a human neurogliaoma cell line, was a gift from the Brain Tumor Research Center at University of California, San Francisco. H4, also a human neurogliaoma cell line, was purchased from American Type Culture Collection and was grown in the presence of 80 μM phorbol ester. Medium conditioned by these cells was collected, Na₂SO₄ was added to a final concentration of 0.02%, and the medium was frozen until use. For each preparation, 3–5 liters of conditioned medium was filtered through Whatman No. 1 paper and concentrated by ultrafiltration through a YM-10 membrane (Amicon). The concentrated conditioned medium was circulated over gelatin-Sepharose to remove MMP-2 and MMP-9, as well as, TIMP-2 in complex with MMP-2 (10). Protein that did not bind the gelatin-Sepharose column, including the small molecular weight inhibitor, was dialyzed against 50 mM Tris, pH 7.5, containing 0.02% Na₂SO₄ and applied to a heparin-Sepharose column. Bound protein was eluted using a gradient of 0 to 1 M NaCl in 50 mM Tris, pH 7.5. Fractions were collected and analyzed for the presence of MMP inhibitors by reverse zymography (18). The small molecular weight inhibitor typically eluted at approximately 625 mM NaCl. These fractions were pooled and passed over a lentil lectin column to absorb TIMP-1, and the flow-through was dialyzed against 50 mM Tris, pH 7.5. The material was then fractionated on a strong cation exchange Sephadex. The concentrated conditioned medium was dialyzed against 50 mM Tris, pH 7.5, and was used to elute the protein. Fractions containing the 16.5-kDa inhibitor protein were identified by SDS-PAGE and by reverse zymography. The small molecular weight inhibitor eluted near the end of the gradient at 1.5 M NaCl. Samples prepared for amino acid sequence analysis were further fractionated by reverse phase liquid chromatography on a C4 column developed with a gradient of CH₃CN in aqueous 0.1% trifluoroacetic acid (10). Samples used to test for enzymatic activity were not passed over the reverse phase column because of the harsh fractionation conditions required. Instead, the small molecular weight inhibitor was purified from the last remaining contaminants by electroelution from SDS-PAGE using an ISCO concentrator. Electroelution was done according to the instructions provided by ISCO.

**Electrophoresis—**Electrophoresis was done according to the method of Laemmli (19) using 15% acrylamide. Modifications to the sample buffer included a 4-fold increase in the amount of SDS and omission of β-mercaptoethanol. Proteins were visualized by staining with Coomassie Brilliant Blue. Inhibitors were visualized using reverse zymography on 15% polyacrylamide gels containing 1% gelatin. After soaking in 2.5% Triton X-100, the gels were soaked in medium conditioned by rabbit synovial fibroblasts treated with phorbol ester (80 μM). This conditioned medium contains a variety of proteinases including several different MMPs. Conditioned medium was removed, and the gel was agitation in substrate buffer (50 mM Tris, pH 8.0, 10 mM CaCl₂, and 0.02% azide) overnight at 0°C. Inhibitor bands were visualized by staining with Coomassie Brilliant Blue followed by destaining with a solution of methanol and acetic acid. To ensure staining was the result of inhibitor protection of the gelatin, a second gel was run in parallel with the reverse zymogram, but soaking in medium conditioned by synovial fibroblasts was omitted.

**Reduction and Alkylation of Inhibitors—**A partially purified fraction of inhibitors from brain tumor conditioned medium (2 mg) was made 4 M with electrophoresis grade urea followed by addition of β-mercaptoethanol (final concentration, 0.14 M). This solution was incubated at room temperature for 2 h. The sample was then alkylated by addition of iodoacetamide (final concentration, 0.7 mM) and incubation in the dark for 20 min at room temperature. The reaction was quenched with β-mercaptoethanol (0.14 M). For the urea control, a sample was made 4 M in urea and incubated in parallel without the addition of β-mercaptoethanol or iodoacetamide. Both samples were dialyzed against 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.02% NaN₃. The effect on inhibitory activity was analyzed by reverse zymography.

**Western Blot Analysis—**After electrophoresis, proteins were transferred to nitrocellulose (20) and probed with antibodies raised against TIMP-1, TIMP-2 (Oncogene/Calbiochem), or TIMP-3 (Chemicon). Anti-peptide antibodies to the C-terminal region of PCPE were raised in rabbits against the peptide GQVEENRGPVL, corresponding to residues 402–412 of the human sequence (21), linked via an aminohexanoic acid spacer to a C-terminal cysteine and coupled to keyhole limpet hemocyanin. Antibodies were affinity-purified on columns of the same peptide coupled to TC gel (Quality Controlled Biochemicals) and the cysteine thiol (22). Secondary antibodies conjugated to horseradish peroxidase, and ECL reagents were purchased from Amersham and used as instructed by the manufacturer.

**Inhibition Assays—**MMP-2 and TIMP-2 were purified from medium conditioned by brain tumor cells according to previously published protocols (10). Reverse phase HPLC was used as the final step to strip TIMP-2 from MMP-2. Removal of TIMP-2, concentration of MMP-2 and repeated freeze thaw cycles of the enzyme resulted in a MMP-2 sample that was largely active as tested by gelatin zymography. CT-PCPE was purified as described above with electroeloration rather than reverse phase HPLC as the final step. The substrate used was the quenched fluorescence peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (Bachem) (23) and was used at a final concentration of 1 μM in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, and 0.02% azide. MMP-2 (20 μM) was mixed with concentrations of either TIMP-2 or CT-PCPE at room temperature followed by the addition of the substrate. Enzyme activity was monitored at 393 nm with excitation at 328 nm using a Perkin-Elmer LS-30 Luminescence Spectrophotometer.

**Expression of Recombinant PCPE—**To produce recombinant human PCPE, a 1370 base pair PCR product was obtained using primers 5'-GATCGGATCCATGCTGCCTGCAGCCACAGCC-3' (forward) and 5'-CTAAGACCTTTACTCTGGACCCAGCAG-3' (reverse), corresponding to nucleotides 1391–1410, respectively, of the reported human PCPE cDNA sequence (Ref. 21; GenBank™ accession number L33799). The ten additional bases of the forward and reverse primers contain BamHI and HindIII recognition sites, respectively. The PCR was performed on a model 480 Thermal Cycler (Perkin-Elmer) under high fidelity/long distance PCR conditions (24, 25) using Taq Extend PCR additive (Stratagene) with denaturing at 94 °C for 3 min, followed by 32 cycles of 98 °C for 7 s with annealing and extension at 68 °C for 3 min. Final incubation was at 70 °C for 5 min. Unamplified cDNA (2 μg) from human dermal fibroblasts (C13DTECH) was used as a template. The PCR product was sequenced on both strands to ensure fidelity, subcloned between the BamHI and HindIII sites of the pFast BacI transfer vector, and then recombined into a baculovirus shuttle vector propagated in Escherichia coli using the Bac-To-Bac system (Life Technologies, Inc.). Resultant recombinant viral genomes were transfection into Sf21 insect cells. Infectious baculovirus were harvested at 72 h and then amplified 48 h in fresh SF21 cells for high titer stocks. Conditioned medium containing approximately 2 μg/ml recombinant PCPE was harvested 4 days after infection of SF21 with high titer stocks. All SF21 cultures were in serum-free SF-900 II/FSM medium (Life Technologies, Inc.).

**Plasmin Degradation of PCPE—**PCPE (270 pmol) was mixed with plasmin in a substrate to enzyme ratio of 37:1 (mol:mol) in 50 mM Tris, pH 7.5. The digestion proceeded for 2.5 h at 37 °C followed by the addition of SDS-PAGE sample buffer. One half of the sample (6.5 μg) was analyzed by electrophoresis on a 15% polyacrylamide gel using a Bio-Rad mini gel apparatus and stained with Coomassie Brilliant Blue. The other half of the sample was analyzed by reverse zymography. To test the activity of plasmin in reverse zymography, the substrate buffer contained 2 mM phenylmethylsulfonyl fluoride. An additional sample was prepared and analyzed by immunoblot using antipeptide antibodies that recognize the C-terminal region of PCPE.

**Amino Acid Sequence Analysis—**Protein to be sequenced was resolved by electrophoresis on a 15% acrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore) (26). Proteins were visualized on the membrane by staining with Coomassie Brilliant Blue. Amino acid sequence analysis was performed by the Biomolecular Resource Center at University of California, San Francisco.
RESULTS

Medium Conditioned by Human Brain Tumor Cells Contains a Metalloproteinase Inhibitor Smaller than the Molecular Weight of Known TIMPs—As previously reported (17), medium conditioned by some human brain tumor cell lines contains at least four inhibitors as detected by reverse zymography. Except for the smallest inhibitor, with an apparent molecular weight of 16,500, the molecular weights of the inhibitors observed by reverse zymography correspond to those of known TIMPs. To examine the proteinase class targeted by the small molecular weight inhibitor migrating at 16,500, medium conditioned by human brain tumor cells was analyzed by reverse zymography. Inhibitor activity was developed in the presence of either the metalloproteinase inhibitor 1,10-phenanthroline (10 mM) or the serine proteinase inhibitor phenylmethanesulfonyl fluoride (2 mM). Phenylmethanesulfonyl fluoride had no effect on the development of inhibitory activity for any of the four brain tumor inhibitors. This indicated that serine proteases were not likely targets for the inhibition observed on reverse zymography (data not shown). However, as expected for the TIMPs, 1,10-phenanthroline interfered with the development of inhibitor activity in reverse zymography. In addition, the presence of 1,10-phenanthroline also interfered with development of inhibitor activity of the 16,500 molecular weight protein. Gelatin substrate was not protected in the presence of 1,10-phenanthroline, indicating that a metalloproteinase was required for the detection of inhibitor bands that are normally detected in the absence of the chelator. Protein amounts of all four inhibitors were not in sufficient quantity to stain through the gelatin background. This was confirmed by running a polyacrylamide gel in parallel with the reverse zymography. The only protein band observed was the contaminant migrating at approximately 43,000 molecular weight (data not shown). This contaminant was also observed to stain through the gelatin background in reverse zymography independent of the absence or presence of 1,10-phenanthroline (Fig. 1). Thus, the 43,000 molecular weight protein was classified as a contaminating protein and not as an inhibitor. Because development of inhibitory activity was sensitive to the presence of 1,10-phenanthroline, the polypeptide migrating at 16,500 molecular weight was designated a metalloproteinase inhibitor.

Several studies have shown the importance of correct disulfide bond pairing for inhibitory activity of TIMP (5–7). To further characterize the small inhibitor, the requirement for disulfide bonds was analyzed. A partially purified protein fraction from human brain tumor conditioned medium containing all four inhibitors was reduced and alkylated under denaturing conditions. Inhibitor function was then analyzed by reverse zymography. Reduction and alkylation completely abolished the inhibitory activity of the known TIMPs as well as that of the 16,500 molecular weight metalloproteinase inhibitor (Fig. 1). However, simply denaturing the sample with urea was not sufficient to produce complete loss of inhibitor function for the TIMPs or for the 16,500 molecular weight inhibitor. These results indicated that, like TIMP, the novel 16,500 molecular weight inhibitor was specific for metalloproteinases and required intact disulfide bonds for inhibitory function.

Inhibitory Activity Observed at 16,500 Molecular Weight Does Not Correlate to a Fragment of a Known TIMP—It has been shown that carboxyl-truncated TIMP-1 and TIMP-2 retain metalloproteinase inhibitory function because inhibitory activity resides in the amino domain of TIMP (27, 28). Because the small inhibitor behaved as a TIMP, the inhibitory activity observed at molecular weight 16,500 on reverse zymography could be derived from a functional TIMP fragment. To test this, Western blot analysis was used to examine a partially purified protein fraction containing all four inhibitor activities.

Antibodies directed against TIMP-1 and TIMP-2 reacted only with proteins corresponding to inhibitors of apparent molecular weights of 29,000 and 20,000, respectively. When this sample was tested for reactivity with anti-TIMP-3 antibody, a protein at molecular weight of approximately 23,000 was recognized, but no reactivity in the range of the small molecular weight inhibitor was observed (Fig. 2). This indicated that the smallest inhibitor was not a fragment of TIMP-1, TIMP-2, or TIMP-3.

Identification of the 16,500 Molecular Weight Inhibitor by Amino Acid Sequence Analysis—To identify the protein responsible for the inhibitory function observed at molecular weight 16,500, inhibitors were isolated using a series of chromatographic steps. The chromatography included: gelatin-Sepharose, heparin-Sepharose, lentil lectin, SP-Sephadex, and reverse phase HPLC. At all steps, column fractions were monitored by SDS-PAGE and reverse zymography. Reverse phase HPLC was used for the final step prior to amino acid sequence analysis. A single protein band was present on SDS-PAGE following HPLC purification (Fig. 3), and this sample was used for amino acid sequence analysis. Although activity of the TIMPs was not affected by exposure to the conditions used in reverse phase HPLC, the activity of the 16,500 molecular weight inhibitor was diminished on reverse zymography after HPLC purification. Therefore, when evaluation of activity was required, the HPLC step was replaced with electrospray. As shown in Fig. 3, electrospray of the 16,500 molecular weight protein from a preparatory electrophoresis gel subsequently produced inhibitory activity when analyzed by reverse zymography. From a typical purification of approximately 5 liters of serum-free medium conditioned by human brain tumor cells, 1.6 mg of active protein was recovered.

From two independent preparations of HPLC purified inhibi-
cells was examined by Western blot and reverse zymography. Lane 1 is partially purified medium conditioned by human brain tumor cells was examined by Western blot and reverse zymography. Lane 1 is reverse zymography showing inhibitory activity. Anti-TIMP-1 (lane 2), anti-TIMP-2 (lane 3), and anti-TIMP-3 (lane 4) signals were developed using ECL reagents. Positions of molecular weight markers are to the left, and the positions of TIMPs are shown at the right.

Fig. 2. Western analysis of brain tumor metalloproteinase inhibitors. Partially purified medium conditioned by human brain tumor cells was examined by Western blot and reverse zymography. Lane 1 is reverse zymography showing inhibitory activity. Anti-TIMP-1 (lane 2), anti-TIMP-2 (lane 3), and anti-TIMP-3 (lane 4) signals were developed using ECL reagents. Positions of molecular weight markers are to the left, and the positions of TIMPs are shown at the right.

The inhibitor activity generated from baculovirus expressed PCPE by plasmin degradation was significant because it occurred in a TIMP-free environment. Thus, this experiment ruled out contamination by a TIMP. These results indicated that although degradation by plasmin generated inhibitor fragments from expressed PCPE, the fragments were not identical to the molecular weights of CT-PCPE purified from brain tumor conditioned medium. Degradation of expressed PCPE by plasmin resulted in some fragments that had inhibitory activity on reverse zymography, but reverse zymography could not establish whether these fragments contained the C-terminal region of PCPE. Western blot analysis was performed on the plasmin digestion mixture by probing with a polyclonal anti-peptide antibody directed to the C-terminal domain of PCPE. As shown in Fig. 6, in addition to the full-length PCPE, the anti-peptide antibody reacted with the protein fragments migrating in the region of 20,000–18,000 molecular weight. The inhibitor observed at a molecular weight of approximately 32,000 in Fig. 5 was not detected with this antibody. It was possible that the concentration of the 32,000 molecular weight fragment was beneath the detection limit of the antibody. It was also possible that the epitope (GQVEEN-RGPVL) used to generate the anti-peptide antibody was not available for binding in this particular fragment. To compare electrophoresis migration positions of inhibitor bands in reverse zymography to Coomassie-stained bands in SDS-PAGE and to immunoreactive bands in Western blot, samples were neither reduced nor boiled. Therefore, it is possible that the epitope in the 32,000 molecular weight fragment was not available for antibody binding. This anti-peptide antibody also reacted with CT-PCPE purified from brain tumor conditioned medium. Consistent with the reverse zymography result, the molecular weights of C-terminal fragments generated by plasmin degradation were slightly larger than that of purified CT-PCPE.

The inhibitor activity generated from baculovirus expressed PCPE by plasmin degradation was significant because it occurred in a TIMP-free environment. Thus, this experiment ruled out contamination by a TIMP. These results indicated that, in vitro, plasmin generated C-terminal fragments from PCPE in the molecular weight range of 20,000–18,000, and some of those C-terminal fragments generated in vitro were metalloproteinase inhibitors as determined by reverse zymography.
Metalloproteinase Inhibitor from Processed PCPE

Fig. 4. Amino acid sequence of human PCPE. Residues 1–449 represent the complete coding sequence of PCPE. The open arrow indicates the beginning of the mature protein. The closed arrows indicate the starting position of each of the five CT-PCPE fragments. The fragments begin at Arg-288, Gly-289 Val-294, Ser-300, and Glu-304. Up to 10 sequence cycles were used to determine identity.

Similarly, it is difficult to determine whether the inhibitory activity is a direct effect or the result of a cascade of multiple proteinases from the same or different proteinase classes. Conditioned medium used to develop the inhibitor bands contains a number of different enzymes, including a number of MMPs (23, 30). MMP-2 is one of the gelatinases that can be present in the conditioned medium used to develop inhibitor bands in reverse zymography. Therefore, CT-PCPE was tested for its ability to inhibit purified MMP-2 in solution using the quenched fluorescent peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 to monitor activity (23).

CT-PCPE was purified from medium conditioned by brain tumor cells as described above with the final step being electrophoresis to preserve activity. The sample was confirmed to be free of contaminating TIMPs by amino acid sequence analysis and by Western blot analysis. As shown in Fig. 7, CT-PCPE fragments inhibited MMP-2 digestion of the peptide substrate. The IC50 value for inhibition was estimated at 560 nM. Using the same assay conditions, an IC50 value of 1.6 nM was calculated for TIMP-2, consistent with values reported for inhibition of MMPs by a TIMP (31). Although not in the same range as TIMP-2, CT-PCPE inhibited MMP-2 in solution. The large IC50 value for CT-PCPE inhibition of MMP-2 may be because MMPs or in particular MMP-2 was not the major metalloproteinase target for CT-PCPE inhibition observed in reverse zymography. Taken together the results presented suggest that CT-PCPE may constitute a new type of metalloproteinase inhibitor.

DISCUSSION

We have identified and partially characterized a small molecular weight metalloproteinase inhibitor observed in conditioned medium by human brain tumor cells. This novel inhibitor with an approximate molecular weight of 16,500 behaves like a TIMP in that it is sensitive to reducing agents and 1,10-phenanthroline interferes with development of its inhibitory activity by reverse zymography. However, inhibitor samples probed with antibody raised against TIMP-1, -2, or -3 failed to recognize a protein in the molecular weight range of 16,500 suggesting that this novel activity was not a TIMP fragment. Amino acid sequence analysis indicated that the 16,500 molecular weight inhibitor was a fragment or a series of fragments containing the C-terminal region of PCPE. Our results cannot rule out the remote possibility that a polypeptide present at a concentration beneath the detection limits of Western blot and amino acid sequence analysis was responsible for the inhibitory activity. However, inhibitor fragments were generated in vitro by plasmin degradation of baculovirus expressed PCPE. Although these fragments were not identical in molecular size to CT-PCPE purified from brain tumor conditioned medium, they were generated in an environment free of TIMP and other mammalian proteins. Taken together, these data support the conclusion that the novel activity observed at 16,500 on reverse zymography is provided by C-terminal fragments of PCPE.
Several different MMPs are present in the conditioned medium used to develop the inhibitor activity bands observed in reverse zymography (23, 30). Although purified native CT-PCPE inhibited MMP-2 in solution, the estimated IC_{50} value (560 nM) was much higher than that determined for TIMP-2 (1.6 nM). This may indicate that MMPs or at least MMP-2 is not the primary target for CT-PCPE inhibition. MMPs other than gelatinases may be the primary target for CT-PCPE inhibition. It is also possible that other metzincins, such as astacins or ADAM, may be the physiological target. However, overlap in specificity may occur. For example, TIMPs appear to inhibit different MMPs with approximately the same kinetics. TIMPs have also recently been shown to inhibit metalloproteinases other than MMPs. An IC_{50} value of 110 nM (0.11 μM) has been reported for TIMP-3 inhibition of TACE (32), and an IC_{50} value of 210 nM has been estimated for TIMP-1 inhibition of aggrecanase (13). Both TACE and aggrecanase are recently identified ADAM family members. Another possible explanation for the large IC_{50} value of CT-PCPE inhibition of MMP-2 in solution is that CT-PCPE may function more efficiently in an insoluble matrix rather than free in solution. This may account for the fact that CT-PCPE can easily be observed by reverse zymography. Still another possibility for the large IC_{50} value is that the sample used for the inhibition of MMP-2 in solution was a pool of CT-PCPE fragments. All of these fragments may not have inhibitory activity. This may also explain the slight difference in the position of the Coomassie Blue-stained band and the position of inhibitor activity in Fig. 3. It is possible that some of the smaller fragments of CT-PCPE have greater inhibitory activity than some of the larger fragments, but the smaller fragments may not be high enough in concentration to be detected by Coomassie Blue staining. This would cause the inhibitor band to appear slightly lower in molecular weight. Because of the heterogeneous nature of CT-PCPE, it will be important to determine the minimum and maximum size required for inhibitor activity. Characterization of the minimum and maximum size required for metalloproteinase inhibition will be essential to design and generate specific CT-PCPE inhibitor fragments that can be used to further characterize this inhibitor.

Based on primary amino acid sequence, CT-PCPE did not share a high degree of identity with TIMP. In mammals, intra-species amino acid identity between TIMPs is low, 34–42%, whereas interspecies identity for a given TIMP is very high. The overlapping functions of TIMPs that show relatively diverse primary structures is attributed to a preserved tertiary structure that is maintained by the conserved positions of 12 cysteine residues. The importance of correctly paired disulfide bonds for TIMP function has been very well documented (5–7). It is possible that CT-PCPE folds in such a way as to present a similar surface to that of TIMP. Evidence that TIMP and CT-PCPE have similar surface charge was observed during the development of a purification method. For example, CT-PCPE co-eluted with TIMP-1 and TIMP-2 from affinity matrices such as heparin-Sepharose and some ion exchange resins. Alternatively, the folding of CT-PCPE may present a disulfide bond pair with neighboring amino acid that can fit into the MMP active site pocket (5). This may account for the similar effects of a reducing agent on CT-PCPE and TIMP function. Using homology search, structure prediction, and structural characterization methods, it was recently proposed that the C-terminal domain of PCPE bears structural homology to the amino domain of TIMP. One striking feature of this comparison is that the disulfide bond pairing in CT-PCPE is predicted to be the same as that in the amino domain of TIMP (33).

PCP was originally isolated from medium conditioned by 3T6 mouse fibroblasts and described as a 55,000 molecular weight protein that enhances the activity of procollagen C-terminal proteinase (PCP) by approximately 10-fold (29, 34, 35). It has been shown that PCP is naturally processed to smaller fragments, some of which also enhance PCP activity (29, 35, 36). Both the 34,000 and 36,000 molecular weight fragments with enhancer function have been mapped to the N-terminal portion of the intact 55,000 molecular weight PCPE (21). The processing of PCP by brain tumor cells may result in two activities, i.e. enhancer function (M_{r} = 34,000 and M_{r} = 36,000) and metalloproteinase inhibitor function (M_{r} = 16,500). We have not detected naturally occurring inhibitory activity associated with the full-length PCPE or with N-terminal fragments. The inhibitory function in the brain tumor cells is associated only with the 16,500 molecular weight CT-PCPE fragment.

There is biological precedence for revealing an activity by proteolytic processing of a larger molecule with an unrelated function. The serine proteinase inhibitor a-1-proteinase inhibitor does not stimulate chemotaxis. However, proteolytic cleavage in the reactive loop of that molecule exposes a 4,200 molecular weight C-terminal fragment that is a potent chemoattractant for human neutrophils (37, 38). More recently, inhibitors of angiogenesis were shown to be proteolytic cleavage fragments. Anti-angiogenic activity is not observed in the full-length molecules, but proteolytic processing of plasminogen and collagen XVIII releases the activities termed angiostatin and endostatin, respectively (39, 40). CT-PCPE appears to be unique in the fact that a cryptic proteinase inhibitor is released upon processing of the full-length PCPE molecule.

PCP, the enzymatic activity enhanced by PCP, has also been identified as BMP-1 (41). The structure of BMP-1/PCP places it in the astacin family of metalloproteinases rather than in the transforming growth factor-β superfamily, as are the other BMP molecules. Many potent morphogenetic molecules including the Drosophila dorsal-ventral patterning gene product tolloid belong to the astacin family and have overall domain structures highly similar to that of BMP-1 (42). The morphogenetic function of BMP-1 may be explained, in part, by the PCP activity (43). However, highly similar molecules, such as tolloid, exert morphogenetic effects in other ways, such as the liberation of transforming growth factor-β-like morphogens from latent complexes (44). Thus, such astacin-like prostates may have pleiotropic effects in morphogenetic processes. The

![Fig. 7. CT-PCPE inhibition of MMP-2. The quenched fluorescent peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ was used to monitor activity of MMP-2. Addition of increasing amounts of electroeluted CT-PCPE to the reaction mixture resulted in a decrease in MMP-2 activity. The open squares represent the means of three different experiments. Error bars represent the S.E. of three different experiments.](image-url)
role of such morphogenetic proteases in neural tissues and brain tumors remains to be determined. As the function of BMP-1 has been redefined by its PCP activity, the function of PCPE may be redefined by its additional activities.

The ability of CT-PCPE to mimic TIMP function suggests that PCPE may play a dual role in collagen deposition: enhancing collagen deposition because of the interaction of the N-terminal domain with PCP and preventing collagen degradation by the inhibition of metalloproteinases such as the MMPs. In both instances, PCPE and CT-PCPE are functioning to facilitate the deposition of collagen. These activities may contribute to fibrosis observed in some circumstances associated with tumor growth. Additionally, the presence of CT-PCPE may correlate with the level of invasive potential of some brain tumor cell lines. Correlation of CT-PCPE with either fibrosis or invasive potential of tumor cells remains to be determined. As the function of PCPE may play a dual role in collagen deposition: enhancing, and for critically reading this manuscript.

Acknowledgment—We thank Dr. Charles Craik for helpful discussions and for critically reading this manuscript.

REFERENCES
1. Stocker, W., Grams, F., Baumann, U., Reinemenn, P., Gemis-Ruth, F.-X., McKay, B. D., and Bode, W. (1995) Protein Sci. 4, 823–840
2. Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson, U. P. (1997) Eur. J. Cell Biol. 74, 111–122
3. Greene, J., Wang, M., Liu, Y. E., Raymond, L. A., Rosen, C., and Shi, Y. E. (1996) J. Biol. Chem. 271, 30375–80
4. Apte, S. S., Mattei, M., and Olsen, B. R. (1994) Genomics 19, 86–90
5. Gemis-Ruth, F.-X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G. P., Bartunik, H., and Bode, W. (1997) Nature 389, 77–81
6. Huang, W., Meng, Q., Suzuki, K., Nagase, H., and Brew, K. (1997) J. Biol. Chem. 272, 22086–22091
7. Caterina, N. C. M., Windsor, L. J., Yermovsky, A. E., Bodden, M. K., Taylor, K. B., Birkenfeld-Hansen, H., and Engler, J. A. (1997) J. Biol. Chem. 272, 32141–32149
8. Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T., and Marmer, B. L. (1992) J. Biol. Chem. 267, 4583–4591
9. Stettler-Stevenon, W. G., Kutzsch, H. C., and Liotta, L. A. (1989) J. Biol. Chem. 264, 17374–8
10. Howard, E. W., and Banda, M. J. (1991) J. Biol. Chem. 266, 17972–17977
11. Howard, E. W., Bullen, E. C., and Banda, M. J. (1991) J. Biol. Chem. 266, 13064–13069
12. Amour, A., Augustin, A., Slocombe, P. M., Webster, A., Butler, M., Knight, C. G., Smith, B. J., Stephens, P. E., Shelley, C., Hutton, M., Knauper, V., Docherty, A. J. P., and Murphy, G. (1998) FEBS Lett. 435, 39–44
13. Arner, E. C., Pratta, M. A., Trzaskos, J. M., Decicco, C. P., and Tortorella, M. D. (1999) J. Biol. Chem. 274, 6594–6601
14. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbassade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wunn, R., Rockwell, A., Yang, P., Duke, J. L., Solomon, K., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, H., Wiswall, B. H., Murphy, K., Hillman, M. C. Jr., Hollis G. F., Newton, R. C., Magdola, R. L., Trzaskos, J. M., and Arner, E. C. (1999) Science 284, 1664–1666
15. Nakano, A., Tani, E., Miyaizaki, K., Yamamoto, Y., and Furuyama, J. (1995) J. Neurosurg. 85, 298–307
16. Matsuzawa, K., Fukuyama, K., Hubbard, S. L., Dirks, P. B., and Rutka, J. T. (1989) J. Neuropathol. Exp. Neurol. 58, 85–96
17. Apodaca, G., Rutka, J. T., Bouhanna, K., Berens, M. E., Giblin, J. R., Rosenblum, M. L., McKerrow, J. H., and Banda, M. J. (1990) Cancer Res. 50, 2322–2329
18. Herron, G. S., Banda, M. J., Clark, R. J., Gavrilovic, J., and Werb, Z. (1996) J. Biol. Chem. 271, 2814–2818
19. Laemmli, U. K. (1970) Nature 227, 680–683
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
21. Takahara, K., Kessler, E., Biniainmonov, L., Brussel, M., Eddy, R. L., Jani-Sait, S., Shows, T. B., and Greenspan, D. S. (1984) J. Biol. Chem. 269, 26280–26285
22. Lee, S., Solow-Cordero, D. E., Kessler, E., Takahara, K., and Greenspan, D. S. (1997) J. Biol. Chem. 272, 19059–19066
23. Murphy, B., and Willenbrock, F. (1995) Methods Enzymol. 248, 496–510
24. Barnes, W. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2216–2220
25. Chang, S., Fuckler, C., Barnes, W. M., and Higuchi, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5695–5699
26. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
27. Miyazaki, K., Funahashi, K., Numata, Y., Koshikawa, N., Akaoi, K., Kikkawa, Y., Yasumitsu, H., and Umeda, M. (1993) J. Biol. Chem. 268, 14387–14393
28. Murphy, G., Houbrechts, A., Cockett, M. I., Williamson, R. A., O’Shea, M., and Docherty, A. J. P. (1991) Biochemistry 30, 8097–8102
29. Kessler, A. M., Mould, A. P., and Hulmes, D. J. (1990) Biochem. Biophys. Res. Commun. 173, 81–86
30. Herron, G. S., Werb, Z., Dwyer, K., and Banda, M. J. (1986) J. Biol. Chem. 261, 2810–2813
31. Lui, Y. E., Wany, M., Green, J., Su, J., Ullrich, S., Li, H., Sheng, S., Alexander, P., Sang, Q. A., and Shi, Y. E. (1997) J. Biol. Chem. 272, 20479–20483
32. Borland, G., Murphy, G., and Agar, A. (1999) J. Biol. Chem. 274, 2810–2815
33. Bando, M. J., and Pathy, L. (1999) Proc. Natl Acad. Sci. U. S. A. 91, 6594–6601
34. Adar, R., Kessler, E., and Goldberg, B. (1986) Colagen Relat. Res. 6, 267–277
35. Kessler, E., and Adar, R. (1989) Eur. J. Biochem. 186, 115–121
36. Hulmes, D. J. S., Mould, A. P., and Kessler, E. (1997) Matrix Biol. 16, 41–45
37. Banda, M. J., Rice, A. G., Griffin, G. L., and Senior, R. M. (1988) J. Exp. Med. 167, 1608–1615
38. Banda, M. J., Rice, A. G., Griffin, G. L., and Senior, R. M. (1986) J. Biol. Chem. 263, 4481–4484
39. O’Reilly, M. S., Shows, T. B., and Greenspan, D. S. (1994) J. Biol. Chem. 269, 32572–32578
40. Reddi, A. H. (1996) Science 271, 463