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TITLE: Novel Breast Tumor Metalloproteinase Inhibitor

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Matrix Metalloproteinases (MMP) are a family of enzymes that degrade the extracellular matrix. Modulation of MMP activity may attenuate the invasiveness of some tumors. MMP activity may also be involved in breast tumor fibrosis because the balance of connective tissue synthesis and degradation is lost. Understanding factors that modulate MMP activity are therefore important to understanding breast tumor biology. We have identified a metalloproteinase inhibitor associated with an invasive breast tumor cell line. The inhibitor was determined not to be a TIMP (Tissue Inhibitor of Metalloproteinase) which are currently the only known metalloproteinase inhibitors. The novel inhibitor (CT-PCPE) was found to be the C-terminal portion of a protein known as Procollagen C-terminal Proteinase Enhancer (PCPE). Intact PCPE has no inhibitor activity. Activity was revealed only by proteolytic processing of the parent PCPE molecule. To investigate CT-PCPE in breast tumors, a plasmid containing the region of CT-PCPE homologous to TIMP was constructed. The structure of expressed CT-PCPE will be compared to TIMP in an effort to better understand CT-PCPE inhibition of MMP. In addition, a proteinase has been observed in the conditioned medium of tumor cell lines that process PCPE to CT-PCPE. Evidence suggests that this may be the proteinase responsible for PCPE processing.
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Introduction

Molecular mechanisms responsible for breast tumor fibrosis and the significance of that fibrosis in breast cancer are poorly understood. It is widely accepted that increased proteinase activity is required for tumor cells to invade and metastasize (Woessner, 1991). Conversely, fibrosis requires excess deposition of connective tissue macromolecules, such as collagen, and the suppression of connective tissue-degrading proteinases, such as Matrix Metalloproteinases (MMP). Collagen deposition, normal or abnormal, involves a series of extracellular molecular events. Nascent collagen must undergo N-terminal and C-terminal processing mediated by a specific procollagen N-terminal proteinase and a procollagen C-terminal proteinase (PCP) (Prockop, et al., 1998). The overall goal of the prosed studies is to determine molecular mechanisms controlling breast tumor connective tissue turnover and to determine the potential of one molecule in particular to affect the course of breast tumor invasion and metastasis. We have identified a novel metalloproteinase inhibitor associated with fatal brain tumors and a highly invasive breast tumor cell line. It is possible that the detection of the molecule could be a prognostic indicator of breast tumor invasion. Procollagen C-terminal Proteinase Enhancer (PCPE) enhances the activity of PCP. However, the enhancer activity resides in the N-terminal domain of the PCPE molecule (Takahara, K. et al., 1994). We have identified a metalloproteinase inhibitory activity associated with the C-terminal domain of PCPE (CT-PCPE). This novel activity is revealed only when the parent PCPE molecule is proteolytically fragmented. This post-translational processing occurs in the extracellular milieu. Post-translational processing may differ slightly between breast and brain tumor cell lines as the functional size of CT-PCPE may be smaller in breast tumor cells. The focus of these studies are to understand the mechanisms by which CT-PCPE is generated from PCPE, to understand the structural requirements for CT-PCPE to function as a metalloproteinase inhibitor and to determine if increased expression of PCPE and/or CT-PCPE correlates with the invasive nature of breast tumor cells.

Body

Task 1  Identification of Functional Domain of CT-PCPE: Previous studies investigating metalloproteinase inhibitors in brain tumor conditioned medium revealed that in addition to the presence of TIMP a fourth activity was observed (Apodaca, et al., 1990). This activity was purified and identified by amino acid sequence analysis. Amino acid sequence analysis indicated that the polypeptide responsible for the inhibitor activity was the C-terminal domain of a protein previously identified as PCPE (Mott et al., 2000). Partial characterization of the inhibitory activity of CT-PCPE against MMP has been examined (Mott et al, 2000). CT-PCPE is also found in the medium conditioned by the breast tumor cell line, MDA MB-231 as determined by reverse zymography and western blot. However, the functional size of the CT-PCPE may differ slightly from that found in the brain tumor conditioned medium. In order to determine the functional size of the CT-PCPE in MDA MB-231 conditioned medium, CT-PCPE was purified using the recently published protocol (Mott, et al., 2000). Briefly, the conditioned medium was passed over gelatin Sepharose in order to remove contaminating gelatinases (MMP-2 and MMP-9). The flow through was fractionated on a heparin Sepharose column. Fractions were analyzed by reverse zymography and those fractions containing the 16.5
kDa inhibitor were pooled and passed over a lentil lectin column. This step removes much of the TIMP-1. Following this the flow through from the lentil lectin was fractionated by SP-Sepharose. The fractions containing the 16.5 kDa were pooled based on the results of reverse zymography. The CT-PCPE was electroeluted from SDS-PAGE to test activity and for amino acid sequence analysis. Results of amino acids sequence analysis have not been conclusive. Although there was only one inhibitor band present by reverse zymography, the amino acid sequence result indicated that there was more than one polypeptide present. Currently, additional preparations of CT-PCPE are being generated in order to obtain a clean amino acid sequence. Information from the amino acid sequence will identify the cleavage sites within PCPE which generate CT-PCPE in the MDA MB-231 cell line.

In addition to directly sequencing purified inhibitor from MDA MB-231 conditioned medium, a molecular biology approach is also underway. We are collaborating with Dr. Richard Williamson of the University of Kent in Canterbury, U.K. to compare the structure of CT-PCPE to TIMP. Dr. Williamson’s laboratory has experience with refolding expressed TIMP molecules and determining their structure with NMR (Williamson et al., 1994). Within the TIMP family of four proteins, the amino acid sequence identity is low. However, the tertiary structural homology is thought to be high due to the conserved placement of twelve cysteine residues. The metalloproteinase inhibitory domain of TIMP resides in the N-terminus of the molecule where six of the twelve cysteines are present. The metalloproteinase inhibitory activity of TIMP is dependent on the integrity of the disulfide bonds as reducing agents such as β-mercaptoethanol abolish this activity (Gomez et al., 1997). CT-PCPE also contains six cysteines and reduction and alkylation abolishes metalloproteinase inhibitor activity (Mott, et al., 2000). Computer modeling suggests that the tertiary structure of CT-PCPE may be homologous to the amino domain of TIMP (Banyai and Patthy, 1999). This could account for the metalloproteinase inhibitory activity of CT-PCPE. Therefore, in collaboration with Dr. Williamson’s group, CT-PCPE will be expressed in bacteria, refolded and its structure determined by NMR. The CT-PCPE structure will be compared to that of TIMP in order to determine if CT-PCPE inhibition of metalloproteinase is due to mimicry of TIMP structure. Additionally, the function of the expressed CT-PCPE will be compared to purified CT-PCPE and to TIMP.

Current progress on this collaboration includes the following. The base pairs coding for amino acids 318-449 of PCPE are predicted to have the greatest similarity to TIMP (Banyai and Patthy, 1999). The bases coding for this region have been cloned by PCR into pPCR-Script (Stratagene). Of several positive clones, two were found to have the correct sized insert by restriction digest. DNA sequencing of both of the clones was performed to insure the integrity of the DNA sequence and the orientation of the insert. Sequencing results indicated that the correct sequence was present in both clones and the insert was present in opposite orientations. Both of these clones have been shipped to Dr. Williamson and we are currently waiting for expression of the CT-PCPE in order to continue these studies.

In order to facilitate the identification and purification of CT-PCPE, an anti-peptide antibody is being generated in rabbits at the Los Alamos National Laboratory in collaboration with Dr. Andrew Bradbury.

Task 2. To determine the biologically relevant proteinases from breast tumor cells that process intact PCPE to the CT-PCPE metalloproteinase inhibitor. Based on the amino termini of the CT-PCPE identified in brain tumor conditioned medium, the amino termini of five of the six fragments have the signature of serine protease cleavage. Moreover, PCPE expressed in a
baculovirus system can be processed in vitro with plasmin to generate smaller fragments of approximately the same size as CT-PCPE. Some of these small fragments have inhibitory activity as determined by reverse zymography (Mott, et al., 2000). Taken together, this information suggests that serine proteinases are most likely the enzymes that process PCPE. Therefore, medium conditioned by MDA MB-231 cells was passed over a lysine Sepharose column. Bound material was eluted with 0.2 M ε-aminocaproic acid. The recovered protein was analyzed by gelatin and casein zymography. A proteinase of apparent molecular weight of 45,000 was observed on both types of zymography (Figure 1). This proteinase was also present in the medium conditioned by brain tumor cells that process PCPE. However, this proteinase was not present in medium conditioned by Hs 27 cells. These cells are normal fibroblasts and CT-PCPE has not been observed in medium conditioned by Hs 27 cells. These data suggest that the 45,000 molecular weight proteinase is an excellent candidate for a PCPE processing enzyme. Future plans regarding this proteinase are to identify it by amino acid sequence analysis and to determine if it can process PCPE to CT-PCPE in vitro.

Task 3: To determine if increased expression of PCPE and/or CT-PCPE correlates with prognosis and the invasive nature of breast tumor cells. MDA MB-231 cells are a highly invasive cell lines which shows signs of epithelial to mesenchymal transition (Sommers et al., 1994 and Gilles and Thompson 1996). The presence and production of CT-PCPE may be specific to cells that are at this late stage of transformation. To test this hypothesis, we are investigating whether different breast tumor cell lines express PCPE and process it to CT-PCPE. The cell lines currently in culture include MCF-7 and MCF-10A. As yet, we have not collected enough conditioned medium from these cell lines to conclude if CT-PCPE is present. However, we have observed CT-PCPE in the medium conditioned by HEK-293 cells (Figure 2). HEK-293 cells are transformed human embryonic kidney cells. As mentioned previously, we have not observed CT-PCPE in the normal human fibroblast cell line Hs 27. Take together, these observations suggest that CT-PCPE may be only expressed and/or processed in transformed epithelial cells. We will also be testing these cell lines for the presence of the 45,000 molecular weight proteinase identified in Task 2.

**Key Research Accomplishments**

1. Construction of two clones to be used in a collaboration with Dr. Richard Williamson to compare the structure of CT-PCPE to TIMP.
2. Identification of a proteinase that may be responsible for the processing of PCPE to CT-PCPE.
3. The finding that CT-PCPE is present in other transformed epithelial cell lines (i.e. HEK-293 cells) and the implication that CT-PCPE may be present in only transformed epithelial cells.

**Reportable Outcomes**

None.
Conclusions

The biology of matrix metalloproteinases and their inhibitors (TIMP) has typically focused on matrix degradation and turnover. These molecules have, therefore, been implicated in fibrotic and metastatic diseases. The cellular origin of these molecules has typically been the fibroblast, other stromal cells, or vascular endothelial cells. However, one of the most striking observations from our research conducted to this point is that the non-TIMP metalloproteinase inhibitor we have designated CT-PCPE appears to be found exclusively in transformed epithelial cells. We have not observed CT-PCPE in normal fibroblast cells nor has its function been observed in the human fibrosarcoma cell line HT-1080. It is also notably absent from human and rabbit vascular endothelial cell. Thus, the production of CT-PCPE from the PCPE may be a marker for transformed and potentially invasive epithelial cells. We will be continuing to examine a number of breast tumor epithelial cell lines both invasive and non-invasive to determine if CT-PCPE can be used as a marker for the invasive nature of breast epithelial cells. Information gathered from this data may be useful in designing assays to screen for CT-PCPE. For example, generating antibodies that detect CT-PCPE in tissue may be useful in screening breast tumors to evaluate their invasive and metastatic potential. This information may be helpful in deciding the course of treatment for specific breast tumors.

In addition to evaluating the correlation of CT-PCPE with the aggressive nature of breast tumor cells, we are continuing to establish the importance of CT-PCPE as a metalloproteinase inhibitor. In an effort to understand the mechanism by which CT-PCPE inhibits MMP, the structure of CT-PCPE is being determined. It has been predicted that CT-PCPE may fold in a similar manner to TIMP. Comparing the two structures by NMR will determine if this is the case. Understanding how the activity of MMPs can be modulated is important in designing compounds that can specifically inhibit MMP. Since tumor cells require the destruction of extracellular matrix in order to invade and metastasize, controlling MMP activity is important for controlling the invasive and metastatic potential of some tumor cells.

References


Prockop, D.J., Sieron, A.L., and Li, S. (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol. 16:399-408.


Figure 1. Zymography of a potential PCPE processing enzyme. Medium conditioned by MDA MB-231 breast tumor cells was passed over a lysine Sepharose column. Material that eluted with ε-aminocaproic acid was analyzed by gelatin and casein zymography. Only one proteinase was present which migrated with an apparent molecular weight of 45,000.
Figure 2. CT-PCPE Inhibitor Activity is Present in Transformed Epithelial Cells. Medium Conditioned by HEK-293 cells (transformed kidney epithelial cells) was concentrated and analyzed by reverse zymography. Both TIMP-1 and TIMP-2 were present as shown above. In addition, an inhibitor which migrated at the position of CT-PCPE (approximately 16.5 kDa) was observed. This inhibitory activity has only been observed in transformed epithelial cells suggesting that it may be a marker for epithelial cell transformation.
Post-translational Proteolytic Processing of Procollagen C-terminal Protease Enhancer Releases a Metalloproteinase Inhibitor*

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Activity of matrix metalloproteinases (MMP) is regulated by a family of proteins called tissue inhibitors of metalloproteinases (TIMP). Four TIMPs have been cloned, and their molecular weights range from 28,000 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 mammary 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)3 (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 TIMPs 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-α 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).

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† The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; PCP, procollagen C-terminal proteinase; PCPE, procollagen C-terminal proteinase enhancer; BMP, bone morphogenic protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction.
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 neuroglioma cell line, was a gift from the Brain Tumor Research Center at University of California, San Francisco. H4, also a human neuroglioma cell line, was purchased from American Type Culture Collection and was grown in the presence of 50 μM phosphotester. Medium conditioned by these cells was collected, NaN₃ 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 YM-10 filter membrane (Amicon). The concentrated conditioned medium was circulated over gelatin-Sepharose to remove MM-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% NaN₃ 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, containing 0.02% NaN₃. 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 lenti lectin column to adsorb TIMP-1, and the flow through was dialyzed against 50 mM Tris, pH 7.5. The material was then concentrated on a strong cation exchange column with IMAC Sephadex A-50. A gradient from 0 to 1.5 M NaCl in 50 mM Tris, pH 7.5, 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 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 inhibitor 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 protocol 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 Coomasie Brilliant Blue. Inhibitors were visualized using reverse zymography on 15% polyacrylamide gels as described previously (10). In brief, samples containing inhibitors were subjected to electrophoresis in 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 proteins including several different MMPs. Coomassie Blue was removed, and the gel was agitated in substrate buffer (50 mM Tris, pH 8.0, 0.1% NaCl, and 0.2% gelatin) overnight at 37 °C. Inducer 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 so that 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 with electrophoresis grade urea followed by addition of β-mercaptoethanol (final concentration, 0.14 M). This solution was then incubated at room temperature for 20 min. The sample was then alkylated by addition of iodoacetamide (final concentration, 0.5 M) 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 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 GQQEERKGPSV, 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) via 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 serum-free conditioned medium of breast 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 electrophoresis rather than reverse phase HPLC as the final step. The substrate used was the quenched fluorescent peptide Mca-Pro-Leu-Glu-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 (final concentration, 2 μM) was mixed with varying concentrations of either TIMP-2 or CT-PCPE at room temperature following the removal of the substrate. The extent of cleavage activity was monitored at 390 nm with excitation at 328 nm using a Perkin-Elmer LS-50 Luminescence Spectrophotometer.

**Expression of Recombinant PCPE**—To produce recombinant human PCPE, a 1370 base pair PCR product was obtained using primers 5-GATGGATGATCAGGCTGCAAGGCTCAGGACCCAGCCT-3 (forward) and 5-CATAGAAGCCGCTACTCTGCTGCAAGCAGCAG-3 (reverse), corresponding to nucleotides 61–81 and 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 Top Extender PCR additive (Stratagene) with denaturing at 94° for 3 min, followed by 32 cycles of 98° for 7 s with annealing and extension at 68° for 3 min. Final incubation was at 70° for 8 min. Unamplified 15 ng of human dermal fibroblasts (CLONTEC) was used as a template. The PCR product was digested with restriction enzymes for high fidelity, subcloned between the BamHI and HindIII sites of the pFastBac transfer vector, and then recombinated into a baculovirus shuttle vector propagated in Escherichia coli using the Bac-To-Bac system (Life Technologies, Inc.). Resultant recombinant viral genomes were transfected into Sf21 insect cells. Infected baculovirus were harvested at 72 h and then amplified 48 h in fresh Sf21 cells in 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 HSFM medium (Life Technologies, Inc.).

**Plasmin Degradation of PCPE**—PCPE (270 pmol) was mixed with plasmin in a substrate to enzyme ratio of 37:1 (mole:mole) 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 μl) was analyzed by electrophoresis on a 15% polyacrylamide gel using a Bio-Rad mini gel apparatus and stained with Coomasie Brilliant Blue. The other half of the sample was analyzed by reverse zymography to diminish 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 antiheparin antibodies that recognize the C-terminal region of PCPE.

**Amino Acid Sequence Analysis**—Protein to be sequenced was re‐isolated by electrophoresis on a 15% acrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-PSQ, Millipore) (26). Proteins were visualized by staining with Coomasie Brilliant Blue. Amino acid sequence analysis was performed by the Biomedical Research Center at University of California, San Francisco.
Fig. 1. Effect of denaturing or reduction and alkylation on inhibitor activity. Reverse zymography of partially purified medium conditioned by human brain tumor cells. Each lane represents 6 µg of protein. Lane 1, untreated; lane 2, incubation in the presence of 10 mM 1,10-phenanthroline; lane 3, exposure to 4 M urea; lane 4, reduced and alkylated. Positions of the molecular weight markers are shown at the right, and positions of the known TIMPs are shown at the left. The arrow shows the position of the novel inhibitor at Mₐ = 16,500.

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 phenylmethylsulfonfyl fluoride (2 mM). Phenylmethylsulfonfyl fluoride had no effect on the development of inhibitory activity for any of the four brain tumor inhibitors. This indicated that serine proteinases 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 inhibitory 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 chromatography 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 electroelution. As shown in Fig. 3, electroelution of the 16,500 molecular weight region from a preparatory electrophoresis gel subsequently produced inhibitor 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 µg of active protein was recovered.

From two independent preparations of HPLC purified inhibi-
Metalloproteinase Inhibitor from Processed PCPE

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.

Fig. 3. Purified 16,500 molecular weight inhibitor. The 16,500 molecular weight inhibitor was purified from medium conditioned by human brain tumor cells. Lane 1 shows a Coomassie Brilliant Blue stain of HPLC purified protein used for amino acid sequence analysis. Lane 2 shows reverse zymography of the 16,500 molecular weight inhibitor purified by electrophoresis. Positions of molecular weight markers are shown on the left, and the expected positions of where TIMP-1 and TIMP-2 would appear, if present, are shown to the right.

ator, five different N termini were detected. All five sequences identified from preparations of the small molecular weight inhibitor matched amino acid sequences near the C terminus of a protein previously described as PCPE (21) (Fig. 4). None of the five sequences contained an N-terminal sequence identical to that of a TIMP. However, comparison of CT-PCPE with the amino domain of TIMP revealed that each contained six cysteine residues. In the case of TIMP, they form three disulfide bonds critical for inhibitory function (5–7). Results shown in Fig. 1 indicated that the six cysteine residues in CT-PCPE may also form disulfide bonds critical for inhibitory function.

Based on the apparent molecular weight of the inhibitor, all five fragments represented polypeptides that contained the majority of the C-terminal domain of PCPE. The N termini of four of the five fragments were consistent with proteolysis by a serine proteinase. These results suggested that the inhibitory activity observed at 16,500 molecular weight was not a TIMP but was C-terminal fragment(s) of PCPE. This activity has been designated CT-PCPE, for C-terminal PCPE. Generation of most of the identified fragments was consistent with processing of PCPE by a serine proteinase that was present either in the conditioned medium or on the surface of the brain tumor cells.

Plasmin Generates Fragments from Expressed PCPE That Show Inhibitor Activity by Reverse Zymography—Amino acid sequence analysis of the CT-PCPE fragments suggested that serine proteinases might be involved in the processing of PCPE. It has previously been shown that trypsin can degrade PCPE in vitro, which releases an active N-terminal region that has PCP/BMP-1 enhancing activity (29). Presumably, a serine proteinase found on the cell surface or one that was secreted into the conditioned medium was responsible for processing PCPE to the inhibitor CT-PCPE. To test whether inhibitory fragments could be released by degradation of PCPE with a serine proteinase, PCPE was expressed in a baculovirus system. Baculovirus was chosen because insect cells do not secrete TIMP. The expressed PCPE protein was digested with plasmin, and the resulting degradation fragments were analyzed by electrophoresis and reverse zymography. The total number of peptide fragments generated by plasmin degradation was examined by staining with Coomassie Brilliant Blue, and inhibitory activity of the fragments was determined by reverse zymography. Thus, by comparing the results of these two techniques, it was possible to determine which fragments were metalloproteinase inhibitors (Fig. 5). Some of the fragments were observed in reverse zymography that did not correspond to those stained with Coomassie Brilliant Blue. This indicated that staining observed by reverse zymography was due to inhibitor protection of the gelatin substrate and not due to polypeptides staining through the gelatin background by non-inhibitory fragments. Several fragments visible by reverse zymography but not visible by simply Coomassie Blue staining were found in the molecular weight range of 20,000–18,000. In addition, one higher molecular weight fragment of approximately 32,000 was observed to have inhibitory activity. Control experiments with plasmin alone did not generate any proteinase inhibitors detected by reverse zymography. Only gelatinolytic bands corresponding to active plasm fragments were visible as cleared areas in the reverse zymography. These results indicated that although degradation by plasmin generated inhibitors 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 (GQLVEEN-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.
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-304, Ser-300, and Glu-304. Up to 10 sequence cycles were used to determine identity.

1 MLFAATASSL GPELLTACALL PFAAQQTQNY TRPVFLCAGD VKGESGYVAS
2 EGPNSTYPN KECITITTVF EQGTVSVLQ VDELEHPPAC RYDALEVFAG
3 SGTSGQRGLR FCQGTRPAFL VAPSNQVTMA MTIDESTTGRF GFLWSYGRSA
4 TSGSEHQCCG GRLEKACQTTL TTPWVNEDS PFSGSCSWHI IAPPQDQIAL
5 TFEKFDFLEPD TCRVYDVSV VFGAVSDDSAR RLSKFCGDAV PGSISSEGNE
6 LLYQFVSALS VTAQGSASAY KTLPRGTAKE QGQPGPKRT EMKVNLLEPSK
7 QPPPEKTEEP SAPDAPTCPE QCRRQSGTLCN NFCASSLVUT ATVCSNMTHP
8 GEGLATVSL ISGAYKTGGCL LPTFPTGSAK LFFYVFCQCPK PKMKGSWYLL
9 MGQVEENKGP VLPQESFVNL HRPQDOQILNT NLSKRRCAPSQ PVRASASQD

Fig. 5. Metalloproteinase inhibitory activity of plasmin processed expressed PCPE. Expressed PCPE (270 pmol) was digested with plasmin (enzyme:substrate = 1:37, mol:mol) for 2.5 h at 37°C. Identical 0.5-µg aliquots of the reaction mixture were analyzed by standard SDS-PAGE to determine the distribution of protein fragments (lanes 1 and 2) and by reverse zymography to detect resulting metalloproteinase inhibitors (lanes 3 and 4). All of the bands with gelatin degrading activity in lanes 3 and 4 can be attributed to fragments of active plasmin. Lane 1 is untreated, expressed PCPE. Lanes 2 and 3 are plasmin processed expressed PCPE. Bands staining darker in lane 3 that in lane 2 represent metalloproteinase inhibitors. Lane 4 is plasmin alone. Molecular weight markers are shown at the left.

1  2  3
43 - 43 -
29 - 29 -
18.4 - 18.4 -
14.3 -

Fig. 6. Immunochemical identification of C terminus of PCPE. Samples containing native and processed PCPE were examined by Western blot using rabbit anti-human anti-peptide antibody raised against the sequence (GQVEENKGPV) present in the C domain of PCPE. The antibody detected inhibitory fragments of CT-PCPE purified from medium conditioned by human tumor cells (lane 1), intact baculo virus expressed PCPE (lane 2), and several bands in the molecular weight range of 20,000-18,000 in plasmin-processed expressed PCPE (lane 3). Signals were developed using ECL reagents. Positions of molecular weight markers are shown at the left.

Native CT-PCPE Inhibits MMP-2 in Solution—CT-PCPE inhibitory activity was originally observed by reverse zymography. Although reverse zymography is a very sensitive technique, specificity of the enzyme target is difficult to determine.

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 was 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 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 inhibitor 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 medium conditioned 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 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, inhibitory 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 (CT-PCPE).
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 acids 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).

PCPE 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 propeptide (PCP) by approximately 10-fold (29, 34, 35). It has been shown that PCPE 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 PCPE by brain tumor cells may result in two activities, i.e. enhancer function ($M_\text{r} = 34,000$ and $M_\text{r} = 36,000$) and metalloproteinase inhibitor function ($M_\text{r} = 16,500$). We have noted 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 α-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 chemottractant 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 PCPE, has also been identified as BMP-1 (41). The structure of BMP-1/PCP places it in the aspartic 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 aspartic 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 asstatin-like proteases may have pleiotropic effects in morphogenetic processes.
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 PCP may be redefined by its additional activities.

The ability of CT-PCP to mimic TIMP function suggests that PCP 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, PCP and CT-PCP 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-PCP may correlate with the level of invasive potential of some brain tumor cell lines. Correlation of CT-PCP with either fibrosis or invasive potential of tumor cells remains to be determined. However, inhibition of metalloproteinases by CT-PCP suggests an alternate mechanism for regulation of proteolytic activity that may be exploited by some tumor cells.

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REFERENCES