Tissue Inhibitor of Metalloproteinase (TIMP-2)
A NEW MEMBER OF THE METALLOPROTEINASE INHIBITOR FAMILY

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Human melanoma cells secrete a 21-kDa protein, termed CSC-21K, which binds with 1:1 molar stoichiometry to the matrix metalloproteinase type IV collagenase proenzyme (70-kDa gelatinase) secreted by the same cells. This binding protein has been purified and its complete primary structure determined by sequencing overlapping peptides which span the entire protein. The amino acid sequence demonstrates that this protein shares significant homology with human TIMP (tissue inhibitor of metalloproteinase), including conservation of the positions of the 12 cysteine residues and 3 of 4 tryptophan residues. The identification of CSC-21K now indicates that a family of TIMP-related proteins exists. Individual members of this family may possess selective affinities for different members of the matrix metalloproteinase family. CSC-21K produced by tumor cells is isolated as a 1:1 molar complex with type IV procollagenase, as demonstrated by amino acid composition analysis. Addition of purified CSC-21K to the activated metalloproteinase results in inhibition of the collagenolytic activity in a stoichiometric fashion. Based on its sequence homology to TIMP and ability to inhibit type IV collagenolysis we propose the name TIMP-2 for this inhibitor.

Matrix metalloproteinases are enzymes capable of degrading both the collagenous and noncollagenous components of the extracellular matrix (1). Their unrestrained activity may result in extensive tissue damage and these enzymes have been implicated in a variety of disease processes, including tumor cell invasion, tumor angiogenesis and rheumatoid arthritis (1-5). Since these enzymes are secreted in zymogen form (6-8), there are at least two possible modes for modulation of their activity within the extracellular milieu. First is the observation of the positions of the 12 cysteine residues and 3 of 4 tryptophan residues. The identification of CSC-21K now indicates that a family of TIMP-related proteins exists. Individual members of this family may possess selective affinities for different members of the matrix metalloproteinase family. CSC-21K produced by tumor cells is isolated as a 1:1 molar complex with type IV procollagenase, as demonstrated by amino acid composition analysis. Addition of purified CSC-21K to the activated metalloproteinase results in inhibition of the collagenolytic activity in a stoichiometric fashion. Based on its sequence homology to TIMP and ability to inhibit type IV collagenolysis we propose the name TIMP-2 for this inhibitor.

Activated interstitial collagenase (14-16). It has been shown that the same cells which produce interstitial collagenase are capable of synthesizing and secreting TIMP (17, 20). Thus net enzyme activity results only when activated collagenase levels exceed inhibitor production. Studies have also shown an inverse correlation between TIMP levels and the invasive potential of murine and human tumor cells (23, 24). Down-modulation of TIMP mRNA levels via antisense RNA resulted in conversion of previously non-tumorigenic and non-invasive Swiss 3T3 cells to tumorigenic cells with invasive properties in vitro and metastatic potential in vivo (25). These data support a link between metalloproteinase regulation and the malignant phenotype.

Recently several new members of the matrix metalloproteinase family have been identified, with various substrate specificities (7, 8, 11, 13, 26, 27). These include stromelysin (homologue of rat transin), type IV collagenase (70-kDa gelatinase) and a 92-kDa gelatinase. While also identified in normal cell types, the overexpression of these enzymes has been linked to malignant conversion and the metastatic phenotype in a number of systems (28-32). Although it has been speculated that other TIMP-like molecules may exist (18, 20, 33, 34), such proteins have not been isolated and sequenced to date. We report here the isolation and sequencing of a novel protein, CSC-21K, which forms a 1:1 complex with type IV procollagenase and inhibits activated type IV collagenase. Binding of CSC-21K to activated type IV collagenase results in inhibition of its collagenolytic activity. Amino acid sequence analysis of CSC-21K, so named for its amino-terminal amino acid sequence and apparent molecular weight on gel electrophoresis, reveals significant homology to TIMP, indicating that CSC-21K is a novel member of the family of TIMP-like proteins.

EXPERIMENTAL PROCEDURES

Culture Methods and Purification of CSC-21K—Human A2058 melanoma cells were grown to 80% confluence in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The medium was then replaced with serum-free Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The medium was then replaced with serum-free Dulbecco's modified Eagle's medium, and the culture continued for an additional 24 h. Approximately 60 liters of human melanoma cell (A2058) serum-free conditioned medium was concentrated to 300 ml using an Amicon YM-30 ultrafiltration membrane. This concentrated conditioned medium was applied to two 1.0 x 10-cm gelatin-Sepharose (Sigma) affinity columns in series, equilibrated with 0.05 M Tris-HCl, 0.5 M NaCl, 0.005 M CaCl2, 0.02% Brij 35, pH 7.6, buffer. The columns were then washed with equilibration buffer before eluting with 0.1% dimethyl sulfoxide in equilibration buffer. The eluate was concentrated and exchanged into 0.05 M Tris HCl, 0.15 M NaCl, 0.005 M CaCl2, 0.02% Brij 35, pH 7.6, using an Amicon YM-30 membrane. The samples were stored at -80 °C.

Samples for anion-exchange chromatography were dialyzed into 0.01 M Tris-HCl, pH 7.5, with 20% ethylene glycol. A 15-μg sample was injected onto a Dionex A1400 HPLC system equipped with a 0.4 x 5.0-cm Dionex ProPac anion exchange column. This column was eluted with a linear gradient of 0-0.4 M NaCl. The material under

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‡The abbreviations used are: TIMP, tissue inhibitor of metalloproteinase; pAPMA, p-aminophenylmercuric acetate.
the single major peak was collected and an aliquot was applied to a 0.46 × 10-cm RP-300 column (Pierce Chemical Co.). This column was eluted at described previously (12). Alternatively, the complex as obtained from gelatin-Sepharose chromatography and stored at −80 °C could be applied directly to the RP-300 column system.

**Enzyme Digestions, Amino Acid Sequencing, and Amino Acid Composition Analyses—**HPLC-purified CSC-21K was reduced and alkylated as described (15). 15 μg of reduced and alkylated CSC-21K was incubated with 5 μg of endoproteinase Lys-C, 5 μg of endoproteinase Arg-C, or 2 μg of endoproteinase Asp-N in 0.1 M NH₂HCO₃ buffer overnight at 37 °C. The digests were then separated by reverse-phase HPLC on the RP-300 column into component peaks which were collected and sequenced individually. Amino acid sequence analysis was carried out on HPLC-purified fractions on a Porton Instruments 2020 gas-phase protein sequenator using standard program 39. Phenylthiohydantoin-derivative identification was carried out on a Beckman System Gold HPLC unit equipped with a 0.46 × 25-cm Beckman ODS column and eluted using a modified sodium acetate/H₄folute/acetonitrile elution method.

**Amino Acid Composition Analyses—**Amino acid composition analyses were performed following vapor-phase hydrolysis for 18 h using 6 N HCl, 0.1% phenol at 120 °C. The hydrolysate was derivatized using the PITC method (PicoTag system, Waters) and analyzed in the same HPLC unit as above using a modified triethylamine/ammonium acetate/acetonitrile elution method.

**Collagenolytic and Gelatinolytic Assays—**Type IV collagenase assays were performed as described previously (7). Gelatinase assays were performed by adaptation of this method utilizing heat-denatured rat skin collagen (Du Pont-New England Nuclear). The CSC-21K collagenase IV proenzyme complex was activated by a 1-h preincubation with 1 mM p-aminophenylmercuric acetate (p-APMA) (12). Subsequently, purified CSC-21K was added prior to the assay of collagenase IV activity.

**RESULTS**

CSC-21K was isolated as a complex with human type IV collagenase by gelatin affinity chromatography of human melanoma cell (A2058) conditioned media. Anion-exchange chromatography of the material obtained from gelatin-affinity chromatography resulted in a single species eluting at approximately 0.18 M NaCl (Fig. 1). Reverse-phase HPLC analysis of material eluted from the ion-exchange chromatography showed that this material contained two components (Fig. 1, inset). The material obtained from the gelatin-affinity chromatography step is thus an intermolecular complex as seen on anion-exchange chromatography, and is not a simple copurification of two species on gelatin-affinity chromatography.

**FIG. 1.** Anion-exchange chromatography of the complex eluted from gelatin affinity chromatography. 15 μg of gelatin-affinity purified material were applied to the anion exchanger. The column was eluted with a linear gradient of NaCl (---). Material from the single major peak eluting at 0.18 M NaCl was rechromatographed on the reverse-phase column (inset). Material from peaks A and B were sequenced directly (see Fig. 2B).

**FIG. 2.** 15% polyacrylamide-SDS gel electrophoresis of CSC-21K and CSC-21K-Type IV collagenase complex (A); aminoterminal amino acid sequences of reverse-phase HPLC peaks (B). Lane A, 2 pg of CSC-21K (Peak A) material following reverse-phase HPLC purification. Lane B, 2 μg of CSC-21K-type IV collagenase complex isolated by gelatin-Sepharose affinity chromatography. The gel was run at 25 mA constant, using a Laemmli sample buffer system and sample buffer containing β-mercaptoethanol. Samples were heated at 95 °C for 2 min prior to electrophoresis. B, the complex obtained following gelatin-affinity and anion-exchange chromatography was further purified into components by reverse-phase HPLC. The materials obtained in Peaks A and B (Fig. 1, inset) were sequenced directly.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the complex obtained from the gelatin-affinity chromatography also showed two components (Fig. 2A). The higher molecular weight material has M, 70,000. It was identified as type IV procollagenase by immunoblotting (data not shown) and amino-terminal sequencing (see below). The lower molecular weight material has an apparent M, of 18,000 which increased to 21,000 upon reduction. Direct reverse-phase HPLC analysis of the complex obtained from gelatin affinity chromatography resulted in the separation of two peaks identical to those in the inset on Fig. 1. The material obtained from each of these peaks, designated peak A for the material with the shorter retention time and peak B for the material of longer retention time, was subjected to amino acid analysis and direct amino acid sequencing. Peak A material gave a unique amino-terminal amino acid sequence shown in Fig. 2B. This material is referred to as CSC-21K. Peak B material gave an amino-terminal sequence identical to latent type IV collagenase (type IV procollagenase) (Fig. 2B).

Amino acid composition analyses of the complex eluted from the gelatin-affinity chromatography and CSC-21K are compared in Table I. The amino acid composition of CSC-21K was isolated as a complex with human type IV collagenase by gelatin affinity chromatography of human melanoma cell (A2058) conditioned media. Anion-exchange chromatography of the material obtained from gelatin-affinity chromatography resulted in a single species eluting at approximately 0.18 M NaCl (Fig. 1). Reverse-phase HPLC analysis of material eluted from the ion-exchange chromatography showed that this material contained two components (Fig. 1, inset). The material obtained from the gelatin-affinity chromatography step is thus an intermolecular complex as seen on anion-exchange chromatography, and is not a simple copurification of two species on gelatin-affinity chromatography.
TIMP-2

TABLE 1

Amino acid compositions of CSC-21K and the CSC-21K-collagenase IV complex

| Amino acid residue | CSC-21K | CSC-21K | Complex |
|-------------------|---------|---------|---------|
|                   | pmol    | residues/mol | pmol    |
| Asp/Asn           | 141     | 18       | 149     |
| Glu/Gln           | 166     | 22       | 127     |
| Ser               | 107     | 14       | 78      |
| His               | 32      | 4        | 25      |
| Gly               | 222     | 29       | 123     |
| Arg               | 56      | 7        | 61      |
| Thr               | 59      | 8        | 86      |
| Ala               | 108     | 14       | 92      |
| Pro               | 93      | 12       | 92      |
| Tyr               | 60      | 8        | 64      |
| Val               | 86      | 11       | 67      |
| Met               | 11      | 1        | 28      |
| Cys               | 33      | 4        | 12      |
| Ile               | 137     | 18       | 83      |
| Leu               | 57      | 7        | 81      |
| Phe               | 50      | 7        | 86      |
| Lys               | 127     | 17       | 107     |

Total 1545 201 1361

Leu/Ile ratio 0.42 0.39 1.02

*Data obtained from direct amino acid composition analysis of reduced and alkylated CSC-21K, or enzyme inhibitor complex as isolated by gelatin-Sepharose chromatography. Samples were hydrolyzed in the vapor phase for 18 h using 6 N HCl, 0.1% phenol at 120 °C before derivitization using the Pico-tag system.

Molar amino acid composition calculated from amino acid composition data assuming 7 phenylalanine residues/mol of CSC-21K.

CSC-21K is significantly different from other collagenase inhibitors (15, 33, 34) and is distinguished by an unusual Leu/Ile ratio. This feature was used to evaluate the stoichiometry of the complex as isolated by gelatin-affinity chromatography. Based on the experimentally determined molar amino acid composition of CSC-21 (7 Leu, 18 Ile, Table I, which is in agreement with the direct amino acid sequence from overlapping peptides, Fig. 4), and the deduced composition of type IV procollagenase (39 Leu/125 Ile, Ref. 13), it was calculated that the theoretical Leu/Ile ratio of a 1:1 molar complex would be 46 Leu/42 Ile or 1.10. This is in excellent agreement with the ratio value of 1.03 that was determined from the amino acid composition analysis of the CSC-21K-type IV procollagenase complex as isolated by gelatin-affinity chromatography. These results also suggest that p-APMA activation of type IV collagenase may be accompanied by the organomercurial-mediated inactivation of CSC-21K; but the results also demonstrate that CSC-21K which has not been exposed to organomercurial compounds is capable of binding to and inhibiting “activated” type IV collagenase.

The complete primary structure of human CSC-21K, determined by sequence analysis of overlapping peptides obtained following endoproteinase Lys-C, endoproteinase Arg-C and endoproteinase Asp-N digestions, is shown in Fig. 4. The amino acid composition of CSC-21K as determined from these sequence data concurs with that obtained by direct analysis of purified CSC-21K (Table I). The molecular weight of CSC-21K calculated from the primary structure is 21,600, which is in good agreement with the gel electrophoresis data. Computer
importing the NBRF-PIR and SWISS-PROT protein sequence data banks (35). Computerized homology searches were applied to the sequences obtained following endoproteinase Arg-C, Lys-C, and Asp-N digestions. The results of these programmed searches are shown. The origin of each of the peptides is identified in the lower half of the figure.

FIG. 4. CSC-21K protein sequence data obtained from the amino terminus and following endoproteinases Lys-C, Arg-C, and Asp-N digestions. Peptide sequences obtained following digests were aligned by overlaps (underlined regions) as shown. The entire sequence of CSC-21K is encompassed by these overlapping peptides. The origin of each of the peptides is identified in the lower half of the figure.

sequences for homology were performed on the BIONET computer were applied to the sequences obtained following endoproteinase Lys-C, Arg-C, and Asp-N digestions. The results of these programmed searches are shown. The origin of each of the peptides is identified in the lower half of the figure.

FIG. 5. Complete sequence for CSC-21K and homology to human TIMP. Computerized homology searches using the BIONET computer were applied to the sequences obtained following endoproteinase Lys-C, Arg-C, and Asp-N digestions. The results of these programmed searches are shown.

These results now indicate the existence of a family of at least two TIMP-like molecules, just as there is a family of metalloproteinases. The metalloproteinase family members have different substrate specificities, and in turn, the members of the TIMP family may show select affinities for members of the metalloproteinase family.

TIMP and TIMP-like inhibitors have been reported to be inactivated by organomercuリアル compounds such as p-APMA by some investigators, but for others, TIMP appeared resistant to inactivation by such compounds (15, 31, 32, 38-41). However, it is clear that CSC-21K which has not been exposed to p-APMA can completely inhibit the activated form of type IV collagenase. Thus CSC-21K is capable of binding to both the latent and activated forms of type IV collagenase, in contrast to TIMP which has been characterized as binding only to the activated form of interstitial collagenase (40, 41). This suggests that there are some inherent differences in the interaction of TIMP with interstitial collagenase compared to the interaction of CSC-21K with another member of this metalloproteinase family.

TIMP is identical to the reported erythroid potentiating activity (EPA) purified from HTLV-II-infected Mo T lymphoblast-conditioned media (22, 42). TIMP also stimulates the proliferation of a human erythroleukemia cell line (K562) and possibly CSC-21K, may regulate other cell functions in addition to extracellular matrix proteolysis.

In summary we have shown that type IV procollagenase secreted from human melanoma cells is complexed to a unique 21-kDa protein which shows significant homology with TIMP. This complex can generate a potent type IV collagenolytic/gelatinolytic activity following treatment with organomercurials in vitro. However, once activated the enzyme activity is susceptible to inhibition by the addition of purified CSC-21K. Based on its ability to inhibit type IV collagenolytic/gelatinolytic activity and its homology to TIMP we propose that this protein be referred to as TIMP-2.

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