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Primary structure and cDNA cloning of human fibroblast collagenase inhibitor

(metalloproteases/glycosylation/peptide:N-glycosidase F/consensus oligonucleotides)

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ABSTRACT **We report the primary structure and cDNA cloning of human fibroblast collagenase inhibitor, a glycoprotein that appears to play a central role in modulating the activity of a number of metalloendoproteases of connective tissue origin including collagenase, gelatinase, and proteoglycanase. Secreted human fibroblast collagenase inhibitor was purified and subjected to automated Edman degradation. The secreted protein consists of 184 amino acid residues; it contains two sites of N-linked oligosaccharide linkage and six disulfide bonds. Synthetic oligonucleotide probes based on selected amino acid sequences of the inhibitor were used to screen a λgt10 cDNA library from a human fibroblast line. Two overlapping cDNA clones were characterized to determine the complete coding and noncoding sequences of the specific mRNA. The amino acid sequence deduced from the nucleotide sequence agrees with that determined by protein sequencing. One clone appears to contain the complete 5′ end and, in addition, the cDNA sequence predicts a 23-amino acid leader peptide. The other clone represents the 3′ end of the mature message and includes a short poly(A)$^+$ tract. This 3′ sequence is remarkably similar to a reported cDNA encoding part of the protein derived from mouse fibroblast poly(A)$^+$ RNA. However, this inhibitor has no substantial homology with previously sequenced protease inhibitors.**

The connective tissue matrix represents a dynamic complex of macromolecules including collagens, elastin, and proteoglycans. Physiological or pathological alterations in this matrix may result from imbalances in the degradation and synthesis of these connective tissue components. Degradation of the major structural protein of this matrix, the interstitial collagens, is initiated by a specific enzyme, collagenase (1–3). A number of other neutral metalloproteinases have also been identified and may be responsible for the degradation of proteoglycans, elastin, and the initial products of collagen degradation (4, 5). Regulation of the activity of these proteinases in the extracellular matrix would seem to be of crucial importance to the organism.

A protease inhibitor designated as human collagenase inhibitor (HCI) appears to play a major role in modulating the activity of interstitial collagenase as well as a number of connective tissue metalloendoproteases (4, 6–8). This inhibitor is a glycoprotein with an apparent mass of 28.5 kDa, which has been purified to homogeneity and biochemically characterized (7). HCI functions through the formation of a tight, 1:1 complex with active collagenase (6, 9). Proteins that are immunologically and functionally identical to dermal HCI have been found in a number of connective tissues, as well as in plasma and amniotic fluid (10). Recently, HCI has been identified as a secretory product of platelets (11) and alveolar macrophages (12).

In this communication, we describe the primary structure of HCI as secreted by human fibroblasts, and the isolation and sequence of the corresponding cDNA clone. The primary structure will aid in understanding the interaction of the inhibitor with its target enzyme and will help indicate which features of the molecule are responsible for its characteristic stability in the face of physical and proteolytic attack. Likewise, the availability of cDNA probes will be invaluable in the study of the *in vivo* regulation of the concentration of this glycoprotein.

MATERIALS AND METHODS

Chymotrypsin, V8 protease, carboxypeptidase Y, and amino acid standard mixtures were purchased from Sigma; endoproteinase Lys-C from Boehringer Mannheim; and peptide:N-glycosidase F from Genzyme (Boston). Iodo-[^{3}H]acetic acid (specific activity, 90 mCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham. All solvents and reagents used for HPLC were obtained from Baker (Phillipsburg, NJ) except trifluoroacetic acid (F_3CCOOH), which was purchased from Pierce. HPLC columns were purchased from SynChrom (Linden, NJ; Synchropak RP-8), Altex (Berkeley, CA; Ultrasphere ODS C_{18}), and IBM (Cyano-RP). The HPLC systems used were from Beckman.

Cell Culture. Normal human skin fibroblasts (CRL-1224) were grown in glass roller bottles as described (3). Human skin fibroblast collagenase inhibitor was purified to homogeneity from the serum-containing medium of these cells as described by Stricklin and Welgus (7).

Normal human embryonic fibroblasts (HEF-SA) were derived from fetal skin tissue and provided by Daniel Rifkin.

Deglycosylation of HCI. Sixty micrograms of native protein was deglycosylated with 1 unit of peptide:N-glycosidase F in 100 μl of 0.2 M sodium phosphate buffer, pH 8.6/10 mM EDTA (13, 14). The reaction mixture was incubated for 12 hr at 37°C.

Proteolytic Digestion of Collagenase Inhibitor. Purified inhibitor was reduced and carboxymethylated (15) using iodo[^{3}H]acetic acid. The alkylated protein was then subjected to a variety of proteolytic digestions. Digestion products were fractionated by reverse-phase HPLC using a Synchropak RP-8 column equilibrated in 0.1% F_3CCOOH (solvent A). Peptides were eluted with a linear gradient of acetonitrile containing 0.1% F_3CCOOH (solvent B). Fractions that proved to contain a mixture of different peptides were repurified by HPLC as described above except that the F_3CCOOH in solvent A and B was replaced by HCl.

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Abbreviation: HCI, human collagenase inhibitor.

Endoproteinase Lys-C digestion. HCI was resuspended in 100 mM NH_4HCO_3, pH 9.0, to a concentration of 0.23 mg/ml and digested with 2.2 units of endoproteinase Lys-C/ml for 17 hr at room temperature.

V8 protease digestion. HCI was resuspended in 50 mM acetic acid, pH 4.0, to a concentration of 1 mg/ml, and digestion with V8 protease was performed at an enzyme-to-substrate ratio of 1:50 (wt/wt) for 18 hr at 37°C.

Chymotrypsin digestion. HCI was resuspended in 1% (wt/vol) NH_4HCO_3, pH 8.0, 0.1 mM $CaCl_2$ to a concentration of 1 mg/ml and digested with chymotrypsin (enzyme-to-substrate ratio 1:50, wt/wt) for 4 hr at 37°C.

Carboxypeptidase digestion. HCI (350 pmols) was resuspended in 50 μl of 100 mM pyridine acetate, pH 5.5. Carboxypeptidase Y was added (enzyme-to-substrate ratio 1:50, wt/wt), and digestion proceeded at room temperature. Samples (10 μl) were withdrawn from the digestion mixture at various time intervals. Amino acids liberated from the carboxyl terminus of HCI were quantitated by phenylthiocarbamoyl amino acid analysis.

Automated Edman Degradation and Phenylthiohydantoin Amino Acid Derivatives Identification. HPLC fractions containing peptides (100–500 pmols) were dried and resuspended in 30–60 μl of solvent containing 15% (vol/vol) F_3CCOOH and 40% (vol/vol) acetonitrile. The peptides were sequenced by automatic Edman degradation using an Applied Biosystems 470A gas-phase sequencer. The resulting phenylthiohydantoin (>PhNCS) amino acid derivatives were dried and resuspended in 25 μl of acetonitrile/water (1:2, vol/vol) containing 62.5 pmols of methyl thiohydantoin tyrosine as internal standard. >PhNCS amino acid derivatives were separated on an IBM 5-μ Cyano-RP column equilibrated in 30 mM sodium acetate, pH 5.1, 5% (vol/vol) tetrahydrofuran (solvent A). Elution was effected by an increasing gradient of acetonitrile (solvent B).

cDNA Synthesis and Molecular Cloning. HEF-SA cells were grown to near confluence in 75-cm^2 tissue culture flasks. Cells were washed twice in Dulbecco's phosphate-buffered saline solution and harvested by the addition of 2 ml of 10 mM Tris·HCl, pH 7.5, containing 1% $NaDodSO_4$, 5 mM EDTA (SET buffer), and 20 μg of proteinase K/ml. Proteinase K was added to the pooled aliquots from the cell harvest to a final concentration of 70 μg/ml and the mixture was incubated at 40°C for 45 min. The proteolyzed solution was phenol:chloroform (1:1; vol/vol) extracted and ethanol precipitated. The precipitated nucleic acids were redissolved in 10 ml of 0.1× SET buffer. RNA was precipitated from this solution by the addition of 10 ml of 4 M LiCl, 20 mM NaOAc, pH 5.0. Poly(A)$^+$ mRNA was isolated using oligo(dT)-cellulose column chromatography as described (16).

Poly(A)$^+$ RNA was primed with oligo(dT) to serve as a template for cDNA synthesis by avian myeloblastosis virus reverse transcriptase as described (17). Following the first strand synthesis, the RNA was hydrolyzed with NaOH. The solution was neutralized, and the cDNA purified by gel filtration chromatography on BioGel A-1.5m in 10 mM Tris·HCl, 5 mM EDTA, and 1% $NaDodSO_4$, pH 7.5. A poly(dG) tail was added to the cDNA by using terminal transferase. Second strand synthesis was primed with oligo(dC) and polymerized in an initial reaction with the large (Klenow) fragment of DNA polymerase. Following second-strand synthesis, *Escherichia coli* DNA polymerase I was added, and incubation was continued to form blunt ends. *Eco*RI restriction sites within the cDNAs were modified by the action of *Eco*RI methylase (18). The cDNA was again purified and ligated to synthetic *Eco*RI linkers. This DNA was ligated into a unique *Eco*RI site in λgt10 DNA packaged *in vitro* and used to infect *E. coli* strain C600 *hfla* 150. Approximately 25,000 recombinants were amplified in this manner.

Recombinant phage were plated at a density of approximately 2×10^3 plaque-forming units per 150-mm Petri dish. Phage were blotted onto nitrocellulose filters, and DNA was denatured and fixed essentially as described (19).

Filters were prehybridized at 37°C in 5× SSPE containing 0.1× SET, 0.15% sodium pyrophosphate, and 1× Denhardt's solutions. (1× SSPE is 10 mM sodium phosphate, 1 mM EDTA, 180 mM sodium chloride, pH 7.4.) Filters were then hybridized for 72 hr at 37°C in this same solution containing 5×10^5 cpm of 5′ end-labeled 51-mer oligonucleotide/ml. Following hybridization, filters were washed six times in 2× SSPE containing 0.1× SET and 0.05% sodium pyrophosphate at 37°C, then three times in 2× SSPE at 21°C. The inserts from two of these clones, λF5 and λF2, were subsequently inserted into bacteriophage M13 and sequenced.

Sequencing was performed by the dideoxynucleotide method (20, 21).

RESULTS

The primary structure of HCI is shown in Fig. 1. The amino acid sequence of amino-terminal residues 1–22 has been published (7). Other peptides originating from digestion of reduced and carboxymethylated HCI were sequenced by automatic Edman degradation to yield the internal sequence. The carboxyl terminus of HCI was determined by digestion of reduced and carboxymethylated protein with carboxypeptidase Y (Fig. 2). Residues 111–118 were not determined

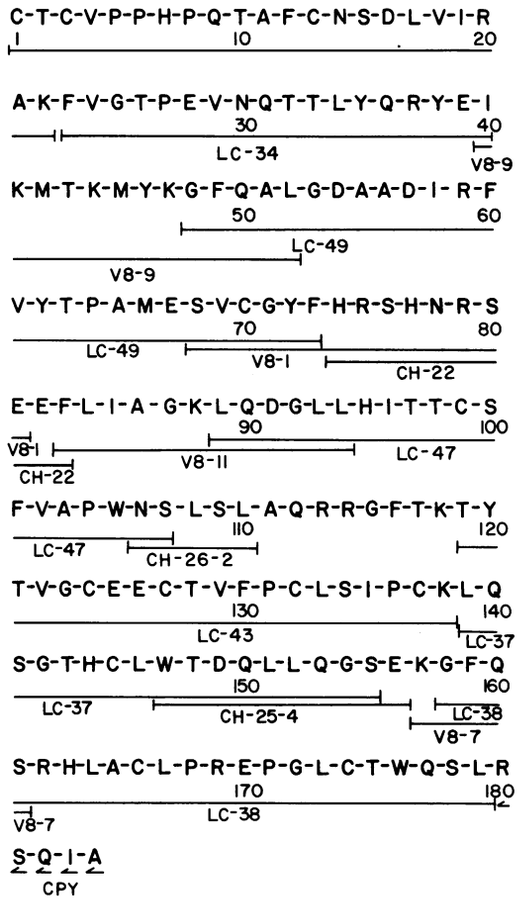

FIG. 1. Primary structure of fibroblast collagenase inhibitor. Selected peptides obtained by enzymatic digestion of the purified protein are shown (LC, endoproteinase Lys-C peptides; V8, staphylococcal protease V8 peptides; CH, chymotryptic peptides; CPY, carboxypeptidase Y).

directly but were derived from the sequence of the inhibitor cDNA as described below.

Finally, several overlaps (residues 22–23, 156–157, 179–180) came from the DNA sequence. Inspection of the HCI amino acid sequence reveals two consensus sequences for sugar attachment, residues 30–32 (-Asn-Gln-Thr-) and residues 78–80 (-Asn-Arg-Ser-). Upon initial sequencing through these regions, the >PhNCS chromatograms for residues 30 and 78 did not reveal any >PhNCS-amino acid derivatives. Deglycosylation of the sugar-containing peptides with peptide:N-glycosidase F prior to sequencing led to the positive identification of residues 30 and 78 as asparagine (detected as >PhNCS-aspartic acid).

Inspection of the primary sequence reveals the presence of 12 cysteines. Since no free sulfhydryls were found in a previous study (7), it appears that a total of six disulfide bonds are present in this protein.

Human fibroblast collagenase inhibitor is a 28.5-kDa glycoprotein based on its electrophoretic mobility in the presence of $NaDodSO_4$ (Fig. 3, lane 2 and ref. 7). Limited exposure of the collagenase inhibitor to peptide:N-glycosidase F results in the generation of two protein species (Fig. 3, lane 3) of approximate molecular sizes of 25 and 20 kDa, respectively. We believe that the upper band is partially deglycosylated while the lower one represents fully deglycosylated HCI. Two pieces of experimental evidence support this view. First, the experimentally determined molecular size of the deglycosylated HCI protein agrees well with the molecular size calculated from the known DNA sequence (see below). Second, recombinant HCI expressed in *E. coli* (and, therefore, nonglycosylated) shows an electrophoretic mobility identical to the 20-kDa HCI species generated by peptide:N-glycosidase F treatment of the native human protein (results not shown).

Approximately 7×10^4 plaques from the amplified cDNA library were screened using the consensus 51-mer oligonucleotide probe shown in Fig. 4. Two "filter lifts" were made of each plate, and signals clearly visible on duplicate filters were used to select phage for plaque purification. Six phage clones were purified and shown to hybridize to the degenerate 17-mer sequence (Fig. 4), which is contained within probe A. Each of the isolates also hybridized to the independent probe C (Fig. 4) (data not shown).

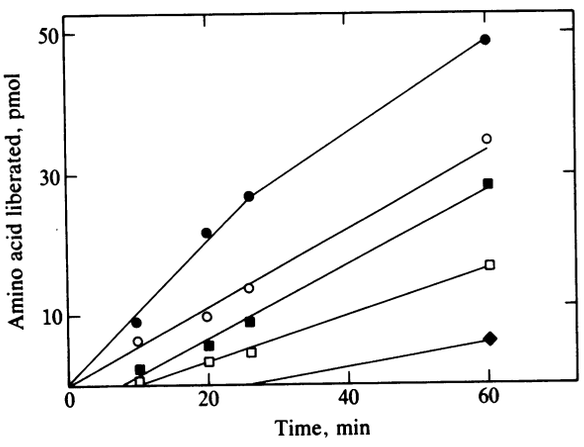

FIG. 2. Carboxypeptidase Y digestion of collagenase inhibitor. Reduced and carboxymethylated inhibitor protein (350 pmols) was digested with carboxypeptidase Y. Samples were removed from the reaction mixture at time intervals shown, and the liberated amino acids were quantitated by phenylthiocarbamoyl amino acid analysis. ●, alanine; ○, isoleucine; ■, glutamine (and some glutamic acid); □, serine; ◆, arginine.

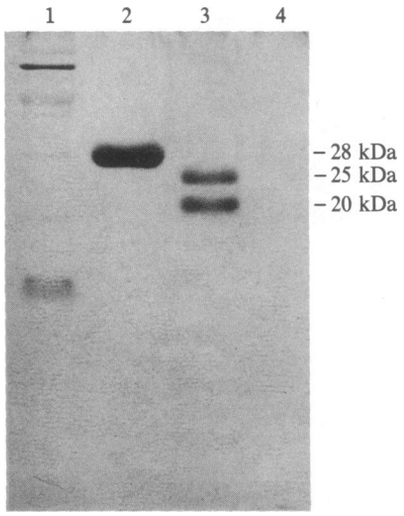

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of native and N-glycanase-treated collagenase inhibitor. Lanes: 1, molecular weight markers; 2, native HCI; 3, HCI treated with 1 unit of peptide:N-glycosidase F; 4, 1 unit of peptide:N-glycosidase F.

DNA was purified from each of these phage, and the inserted cDNA was released by *Eco*RI digestion. Internal *Eco*RI sites were not found in any of the inserts. Using the insert from one of these clones, F5, as a probe, we showed by Southern analysis that each of the other clones contained homologous sequences. Clones F5 and F2 were characterized further.

A restriction map of clones F2 and F5 was generated with respect to the enzymes *Pst* I, *Eco*RI, and *Nco* I and is shown in Fig. 5. As can be seen, F2 and F5 each contain unique sequences. Inserts from both clones were inserted into the *Eco*RI site of M13 phage vector mp19 for sequence determination. In addition, *Pst* I–*Pst* I and *Eco*RI–*Pst* fragments of insert F5 were inserted into mp18. The sequencing strategy is shown in Fig. 5. In two instances, primer oligonucleotides were synthesized to facilitate sequencing. The complete nucleotide sequence, which is a composite of data from clones F2 and F5, is shown in Fig. 6. The sequence is comprised of 811 nucleotides, including homopolymeric tracts and *Eco*RI linkers at either end, consistent with the cloning methodology. The poly(A) tract at the 3′ end is separated by 15 bases from the consensus sequence AATAAA, which is characteristic of the *in vivo* polyadenylylation site of eukaryotic mRNA (23). The poly(dC) observed at the 5′ end of the sequence is expected from the procedures used to construct the cDNA library. Whether some portion of this tract represents cytidine residues that are present in the mRNA is not clear. It seems likely that the 48-nucleotide region preceding the first ATG represents most, if not all, of the 5′-nontranslated region present in the inhibitor mRNA. More precise mapping experiments are necessary to address the point with certainty.

An open reading frame is observed for the 618 bases following the first ATG. Translation in this frame prescribes an amino acid sequence that is in agreement with the protein structure determined by Edman degradation. In addition, this translation frame shows a 23-amino acid extension at the amino terminus of the mature inhibitor. This extension harbors the features commonly found in leader peptides of secreted proteins (24). These include a preponderance of uncharged and hydrophobic amino acids and a cleavage site immediately following an alanine.

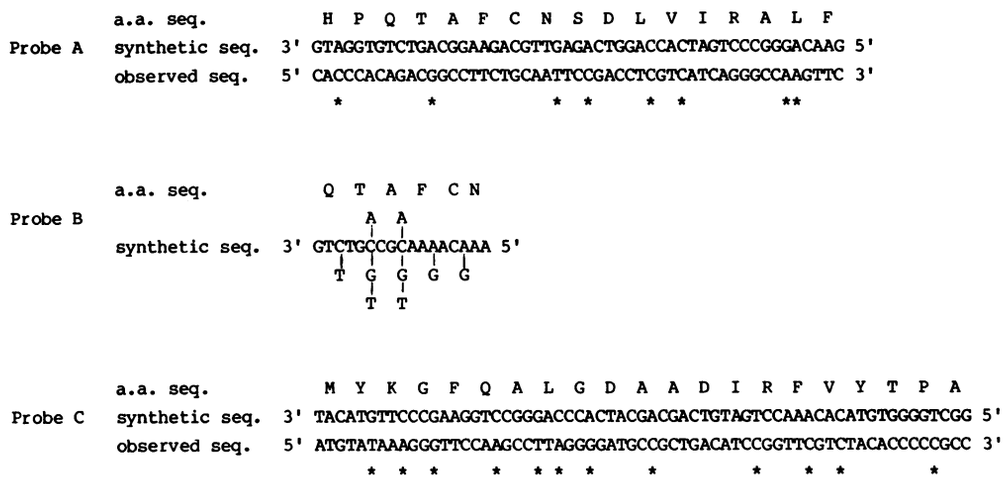

FIG. 4. Oligonucleotide probes. The three oligonucleotide probes were based on the amino acid sequences shown. Codon choices for consensus probes A and C were based on those most frequently observed in mammalian genes (22) and altered where necessary to alleviate potential intrastrand base pairing. The observed sequence in the cDNA is also shown. Mismatches between the predicted and observed sequences are indicated with an *. Probe B contained all 128 possible sequences encoding the hexapeptide shown.

A comparison of the inhibitor cDNA sequence with sequences in the Genetic Sequence Data Bank[§] shows a substantial homology with a cDNA clone containing the partial sequence isolated from a mouse fibroblast cDNA library (25). The extensive homology with the present collagenase inhibtor at both the nucleotide and amino acid level suggest that the clone isolated by these authors encodes an analogous metalloproteinase inhibitor for the mouse.

DISCUSSION

Human fibroblast collagenase inhibitor is a glycoprotein that appears to be ubiquitous in human mesodermal tissues (7, 10). The role of this protein as an inhibitor of interstitial collagenase is clear (6, 7, 9), and the inclusion of several other metalloproteases within its inhibitory spectrum is evident (4, 8). Both biochemical and immunologic studies indicate that HCI is identical to the tissue inhibitor of metalloproteases (TIMP) described by others (8).

The native protein has an apparent molecular mass of 28.5 kDa as estimated by $NaDodSO_4$ gel electrophoresis. Removal of the carbohydrate by N-glycanase digestion alters the mobility of the protein in $NaDodSO_4$/polyacrylamide gel electrophoresis to suggest that the molecular mass of the core protein is approximately 20 kDa, which agrees with 20.711 kDa calculated from the amino acid composition.

The amino acid sequence of the mature protein has been largely determined by Edman degradation of purified peptides. The sequence is corroborated by the cDNA sequence presented. Amino acids -111 to -118 are predicted by the cDNA sequence and are consistent with the amino acid composition of both purified protein and derived peptides but have yet to be sequenced.

Two consensus sequences for N-linked carbohydrate attachment are identified. Based on the sequence analysis of these peptides before and after peptide:N-glycosidase F treatment, it appears that both sites are modified asparagines in the native protein. The presence of a single intermediate in the enzymatic deglycosylation is consistent with two glycosylated residues. More extensive treatment with peptide:N-glycosidase F completely converts the protein to the fastest migrating species (ca. 20 kDa) without inactivating the inhibitory activity of HCI (G.P.S., unpublished observations).

The 3′ end of the cDNA sequence presented is remarkably similar to a previously reported partial cDNA generated from mouse fibroblast poly(A)$^+$ RNA (25). The homology between

[§]National Institutes of Health (1983) *Genetic Sequence Databank: Genebank* (Research Systems Div., Bolt, Beranek, and Newman Inc., Boston), Tape Release 15.0.

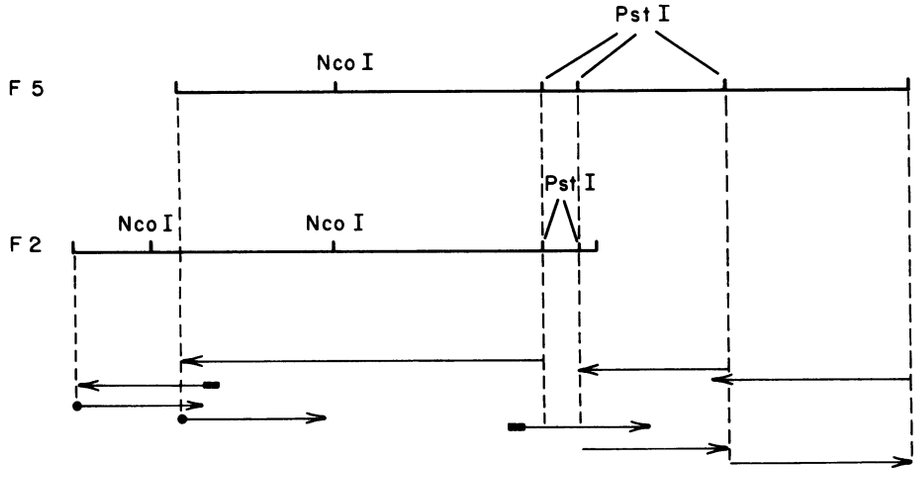

FIG. 5. Restriction map and sequencing strategy for cDNAs contained in clones F2 and F5. Insert DNA from λ clones F2 and F5 and fragments of F5 generated by *Eco*RI and *Pst* I double digests were inserted into M13 for sequencing. Arrows indicate the direction and length of the sequence analyses. Sequencing the 5′ end of each clone required transcription through single-stranded poly(dG) tracts. These were initiated by primers containing $(dC)_8$ at the 3′ end and are indicated by closed circles. Synthetic primers complementary to cDNA sequences are represented by closed boxes.

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                                      30                                      60
GAA TTC CCC CCC CCT CCC CCC CCC GGC CAT CGC CGC AGA TCC AGC GCC CAG AGA GAC ACC

                                      90                                     120
AGA GAA CCC ACC ATG GCC CCC TTT GAG CCC CTG GCT TCT GGC ATC CTG TTG TTG CTG TGG
                Met Ala Pro Phe Glu Pro Leu Ala Ser Gly Ile Leu Leu Leu Leu Trp

                                     150                                     180
CTG ATA GCC CCC AGC AGG GCC TGC ACC TGT GTC CCA CCC CAC CCA CAG ACG GCC TTC TGC
Leu Ile Ala Pro Ser Arg Ala Cys Thr Cys Val Pro Pro His Pro Gln Thr Ala Phe Cys

                                     210                                     240
AAT TCC GAC CTC GTC ATC AGG GCC AAG TTC GTG GGG ACA CCA GAA GTC AAC CAG ACC ACC
Asn Ser Asp Leu Val Ile Arg Ala Lys Phe Val Gly Thr Pro Glu Val Asn Gln Thr Thr

                                     270                                     300
TTA TAC CAG CGT TAT GAG ATC AAG ATG ACC AAG ATG TAT AAA GGG TTC CAA GCC TTA GGG
Leu Tyr Gln Arg Tyr Glu Ile Lys Met Thr Lys Met Tyr Lys Gly Phe Gln Ala Leu Gly

                                     330                                     360
GAT GCC GCT GAC ATC CGG TTC GTC TAC ACC CCC GCC ATG GAG AGT GTC TGC GGA TAC TTC
Asp Ala Ala Asp Ile Arg Phe Val Tyr Thr Pro Ala Met Glu Ser Val Cys Gly Tyr Phe

                                     390                                     420
CAC AGG TCC CAC AAC CGC AGC GAG GAG TTT CTC ATT GCT GGA AAA CTG CAG GAT GGA CTC
His Arg Ser His Asn Arg Ser Glu Glu Phe Leu Ile Ala Gly Lys Leu Gln Asp Gly Leu

                                     450                                     480
TTG CAC ATC ACT ACC TGC AGT TTC GTG GCT CCC TGG AAC AGC CTG AGC TTA GCT CAG CGC
Leu His Ile Thr Thr Cys Ser Phe Val Ala Pro Trp Asn Ser Leu Ser Leu Ala Gln Arg

                                     510                                     540
CGG GGC TTC ACC AAG ACC TAC ACT GTT GGC TGT GAG GAA TGC ACA GTG TTT CCC TGT TTA
Arg Gly Phe Thr Lys Thr Tyr Thr Val Gly Cys Glu Glu Cys Thr Val Phe Pro Cys Leu

                                     570                                     600
TCC ATC CCC TGC AAA CTG CAG AGT GGC ACT CAT TGC TTG TGG ACG GAC CAG CTC CTC CAA
Ser Ile Pro Cys Lys Leu Gln Ser Gly Thr His Cys Leu Trp Thr Asp Gln Leu Leu Gln

                                     630                                     660
GGC TCT GAA AAG GGC TTC CAG TCC CGT CAC CTT GCC TGC CTG CCT CGG GAG CCA GGG CTG
Gly Ser Glu Lys Gly Phe Gln Ser Arg His Leu Ala Cys Leu Pro Arg Glu Pro Gly Leu

                                     690                                     720
TGC ACC TGG CAG TCC CTG CGG TCC CAG ATA GCC TGA ATC CTG CCC GGA GTG GAA GCT GAA
Cys Thr Trp Gln Ser Leu Arg Ser Gln Ile Ala End

                                     750                                     780
GCC TGC ACA GTG TCC ACC CTG TTC CCA CTC CCA TCT TTC TTC CGG ACA ATG AAA TAA AGA

                                     810
GTT ACC ACC CAG CAA AAA AAA AAA GGA ATT C
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FIG. 6. The complete cDNA sequence and predicted translation product compiled from clone F2 and F5 are shown. The consensus signal for polyadenylylation, nucleotides 773–778, is underlined. It is less clear that the 5′ end of the mRNA is completely represented by the sequence shown. The predicted translation product agrees completely with the observed sequence as presented in Fig. 2, with the exception of the amino acids, which were not sequenced but which are prescribed by nucleotides 479–495. In addition, the cDNA sequence depicts a 23-amino acid leader peptide characteristic of secretory preproteins.

the predicted translation products is 69% for the 39-carboxyl-terminal amino acids. Moreover, the homology continues into the 100 bases of the 3′-untranslated end where 68% conservation of structure is found. Such a pattern of conserved sequences in the 3′-untranslated region are frequently observed in homologous proteins from different species (26).

No pronounced sequence homology was found between this inhibitor and any previously described protease inhibitors. It represents a distinct class of inhibitor protein, which is consistent with its being the first metalloprotease inhibitor sequenced. It is possible that other members of this class exist. However, analysis of total poly(A)$^+$ RNA from human fibroblasts on RNA blots has failed to detect related RNAs, even at reduced hybridization stringencies (C. Roberts and D.F.C., unpublished observations).

The sequence information provided in this communication is an important step toward understanding the interaction between the inhibitor and its target protease. Additionally, the cDNA structure described herein will provide a new tool for studying the regulation of the biosynthesis of the inhibitor. Clearly, an orchestrated regulation of the tissue protease(s) and inhibitor(s) must exist to preserve or remodel the extracellular architecture as necessary. It is expected that the availability of molecular probes will assist in the understanding of these processes.

Note Added in Proof. Following submission of this manuscript, a cDNA encoding TIMP was reported (27). It is now clear that TIMP and HCI are identical to each other and to the protein originally identified as an erythroid-potentiating activity (28).

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