Cloning of a 72 kDa matrix metalloproteinase (gelatinase) from chicken embryo fibroblasts using gene family PCR: expression of the gelatinase increases upon malignant transformation

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Chicken embryo fibroblasts secrete a 72 kDa progelatinase that displays all of the characteristics of a matrix metalloproteinase. Employing reverse-transcription PCR and degenerate oligonucleotide primers that are specific for two highly conserved sequences found in all matrix metalloproteinases, a DNA fragment specific for the chicken gelatinase was generated. Using this PCR product as a probe, cDNA clones were isolated from a chicken embryo cDNA library and the entire protein coding sequence was determined. The chicken progelatinase is 84% identical, at the amino acid level, with human and mouse 72 kDa progelatinase/type-IV procollagenase, with the greatest degree of similarity occurring in the propeptide and catalytic domains. The avian and mammalian proteinases diverge significantly in the C-terminal, hemopexin-like domain. The last 100 residues of the chicken gelatinase are only 66% identical with mammalian

gelatinases. Mouse 72 kDa progelatinase, however, does not diverge significantly (> 98% identity) from human progelatinase in the hemopexin-like domain. The divergence in this domain of the chicken progelatinase may explain some of the distinct catalytic and inhibitory properties of the 72 kDa chicken progelatinase. Northern-blot analysis reveals that steady-state levels of the chicken progelatinase mRNA are increased 5-fold upon malignant transformation of chicken embryo fibroblasts with Rous sarcoma virus (RSV) and 3-fold by treatment with the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA). This represents the first reported cloning of an avian matrix metalloproteinase. The increased expression of the chicken progelatinase by RSV transformation and the tumour promoter PMA suggests that the progelatinase is regulated differently in chicken cells.

INTRODUCTION

Extracellular matrix (ECM) remodelling represents an important event in many normal and pathological processes. One major proteinase family, the matrix metalloproteinases (MMPs), appear to play a central role in degrading components of the ECM. Their functions in embryo implantation (Librach et al., 1991), inflammatory diseases (Case et al., 1990), angiogenesis and tumour invasion and metastasis (Liotta et al., 1991) have been well documented. Several members of this proteinase family have been described and include: two collagenases (MMP-1 and MMP-8) (Macartney and Tschesche, 1983; Goldberg et al., 1986); two gelatinases/type-IV collagenases (MMP-2 and MMP-9) (Collier et al., 1988; Wilhelm et al., 1989); three stromelysins (MMP-3, -10 and -11) (Chin et al., 1985; Matrisian et al., 1985; Nicholson et al., 1989; Basset et al., 1990); matrilysin (MMP-7) (Woessner and Taplin, 1988); and a recently cloned mouse macrophage elastase (Shapiro et al., 1992). Individual members of the MMP family degrade various matrix proteins, including interstitial and basement membrane collagens, fibronectin, elastin and proteoglycan core protein (Docherty and Murphy, 1990).

Although their substrate specificities and patterns of expression vary, MMPs share functional and structural features. These proteinases function at neutral pH values, are inhibited by a family of protein inhibitors [the tissue inhibitors of

metalloproteinases (TIMPs)] (Winyard et al., 1991), require an intrinsic zinc ion for catalytic activity, and are secreted as inactive zymogens (Woessner, 1991). A highly ordered domain structure confers both the shared and unique properties to each of these enzymes. All MMPs contain at least two domains: a propeptide and a catalytic domain. In addition, a large C-terminal domain, which shows some sequence similarity to hemopexin (Muller et al., 1988) and the ECM-protein vitronectin (Matrisian et al., 1986), is present in all members except MMP-7 (Quantin et al., 1989). The catalytic domains of MMP-2 and MMP-9 are interrupted by a large fibronectin-like gelatin-binding domain and MMP-9 contains a small type-V collagen-like domain.

A well-studied member of the MMP family is MMP-2, which has been described in humans and other mammals as well (Seltzer et al., 1981; Salo et al., 1983; Murphy et al., 1985). Human MMP-2 was shown to cleave gelatin (denatured collagen) with high specific activity and basement-membrane type-IV collagen at a reduced, yet significant rate (Collier et al., 1988). Considerable controversy still exists over the ability of MMP-2 and MMP-9 to cleave native type-IV collagen; some studies have demonstrated that these gelatinases are weak type-IV collagenases (Mackay et al., 1990; Okada et al., 1990; Nagase et al., 1991; Morodomi et al., 1992). Recently our laboratory has reported the isolation and characterization of a gelatinolytic

Abbreviations used: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; CEF, chicken embryo fibroblast; RSV, Rous sarcoma virus; RT, reverse transcription; SSPE, (0.18 M NaCl/10 mM Na₂HPO₄, pH 7.4/1 mM EDTA); CG62, degenerate oligonucleotide to the N-terminus of the active chicken gelatinase; APMA, *p*-aminophenylmercuric acetate; M-MuLV, Moloney murine leukaemia virus; ECM, extracellular matrix; PMA, phorbol 12-myristate 13-acetate.

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The nucleotide sequence data reported will appear in GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U07775.

enzyme secreted by Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (CEF) that appears to be a member of the MMP family (Chen et al., 1991). Partial N-terminal sequencing and the presence of a 21 kDa protein that co-purifies with the chicken progelatinase suggested that this enzyme is an avian homologue of human MMP-2. Although this gelatinase rapidly degrades denatured collagens it does not appear to cleave native type-IV collagen (Chen et al., 1991). A direct comparison with MMP-2 proteinases in various other species may help to elucidate the basis for these differences in substrate specificity and may also help reveal the basis of substrate specificity in all of the MMP-family members.

Two separate sequences are believed to be responsible for the co-ordination of the catalytic zinc ion in the MMP zymogens: the cysteine-switch and the zinc-binding regions (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990; Windsor et al., 1991). The sequence conservation between the MMPs in these regions is remarkable and was exploited to clone the cDNA encoding the chicken progelatinase. A similar approach has been utilized successfully to identify serine- and cysteine-proteinase cDNAs from the parasite *Anisakis simplex* (Sakanari et al., 1989). Comparison of the nucleotide and deduced amino acid sequences of the chicken cDNA clone with those of the mammalian proMMP-2 reveals a high level of identity. Interestingly, this identity is not distributed homogeneously over the entire molecule, as chicken and human MMP-2 appear to diverge significantly at the C-terminus. This divergence is interesting because this region of human MMP-2 has been shown to be crucial for TIMP binding (Murphy et al., 1992b) and has been implicated in the substrate specificity of collagenase (Murphy et al., 1992a; Sanchez-Lopez et al., 1993).

MATERIAL AND METHODS

Materials

Pathogen-free, complement fixation avian leukosis (COFAL)-negative eggs were purchased from SPAFAS (Norwich, CT, U.S.A.). LiCl, urea, cell culture reagents, ethidium bromide, Tris, EDTA, sodium citrate and NaCl were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Phenol, formamide, nucleic acid molecular-mass markers, T4 polynucleotide kinase, sequencing primers and restriction endonucleases were purchased from BRL (Grand Island, NY, U.S.A.). Radiolabelled deoxyribonucleotides were purchased from Amersham (Arlington Heights, IL, U.S.A.). Moloney murine leukaemia virus (M-MuLV) reverse transcriptase, and random prime DNA labelling reagents were purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Reagents for PCR were purchased from Perkin-Elmer Cetus (Norwalk, CT, U.S.A.). Magna nylon membranes were from Micron Separations (Westboro, MA, U.S.A.). Methylene Blue stain was obtained from Molecular Research Center (Cincinnati, OH, U.S.A.). Erase-a-base nested deletion kit and Magic miniprep kits were purchased from Promega (Madison, WI, U.S.A.). The T/A cloning kit was from Invitrogen (San Diego, CA, U.S.A.). DNA-sequencing reagents were purchased from United States Biochemical (Cleveland, OH, U.S.A.). Fetal bovine serum was purchased from Hyclone (Logan, UT, U.S.A.). A 10-day-old chicken embryo λ gt11 cDNA library was purchased from Clontech (Palo Alto, CA, U.S.A.). Oligonucleotides were obtained from either Operon Technologies (Alameda, CA, U.S.A.) or Genosys Biotechnologies (The Woodlands, TX, U.S.A.). pBlue-script II KS⁺ was purchased from Stratagene (La Jolla, CA, U.S.A.) and phorbol 12-myristate 13-acetate was obtained from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.).

Cell culture

Primary cultures of CEF were prepared from 10-day-old pathogen-free, COFAL-negative fertilized eggs as previously described (Fairbairn et al., 1985; Sullivan and Quigley, 1986; Chen et al., 1991). Secondary cultures of CEF were infected with RSV as described previously (Sullivan and Quigley, 1986; Chen et al., 1991). Alternatively, non-transformed cells were maintained essentially as above without infection. PMA was included in culture medium at a concentration of 100 ng/ml where indicated.

RNA isolation and analysis

Total cellular RNA was isolated from cells using a modified LiCl/urea extraction procedure (Auffray and Rougeon, 1980; Bothwell et al., 1990). RNA was dissolved in 10 mM Tris (pH 7.5)/1 mM EDTA (pH 8.0). Northern blots were performed using formaldehyde/agarose denaturing gels (Maniatis et al., 1982). After electrophoresis, RNA was transferred to nylon membranes and was stained with Methylene Blue (Maniatis et al., 1982) following the manufacturer's recommendations to verify equal loading. Membranes were prehybridized in 50% (v/v) formamide/5 $\times$ Denhardt's solution [1 $\times$ Denhardt's = 0.02% BSA serum albumin/0.02% Ficoll 400/0.02% poly(vinylpyrrolidone)]/5 $\times$ SSPE [1 $\times$ SSPE = 0.18 M NaCl/10 mM Na₂HPO₄ (pH 7.4)/1 mM EDTA]/0.2% SDS/100 μ g/ml salmon sperm DNA at 42 °C and hybridized with DNA probes labelled with [α -³²P]-dCTP using random priming. Blots were washed in a 0.2 $\times$ SSPE/0.1% SDS solution and exposed to Fuji XAR film.

DNA isolation and analysis

Plasmid DNA was isolated using a Magic miniprep kit according to the manufacturer's recommendations. λ phage DNA was isolated using poly(ethylene glycol) precipitation as previously described (Maniatis et al., 1982). For Southern-blot analysis (Southern, 1975), gels were denatured, neutralized and the DNA was transferred to nylon membranes by capillary blotting. Oligonucleotide probes were hybridized in 1 $\times$ SSPE/5 $\times$ Denhardt's solution/100 μ g/ml salmon sperm DNA at 25 °C. Blots were washed in 0.2 $\times$ SSPE/0.1% SDS as indicated.

Labelling of DNA probes

Oligonucleotides were end-labelled with [γ -³²P]dATP using T4 polynucleotide kinase as previously described (Maniatis et al., 1982). Larger probes were random prime labelled with [α -³²P]dCTP according to the manufacturer's protocol.

Oligonucleotide construction

Three degenerate oligonucleotides were used to clone the cDNA encoding the 72 kDa chicken gelatinase. Two of these were used as primers for PCR and were constructed to hybridize to portions of the nucleotide sequences that encode the cysteine-switch region (5' primer) and the zinc-binding region (3' primer). The sequence of the 5' primer is 5'-GATCGAGCTCTGYGG-NAAYCCNGAYGT-3' (degenerate nucleotide positions are indicated by the standard IUPAC one-letter code. Y = T or C; R = A or G; D = A, G or T; M = A or C) and contains the nucleotide sequence that encodes the consensus amino acid sequence C⁷³GNPDV. The 3' primer sequence is 5'-CGACGTCGACTGNCCRAAYTCRTGDGC-3' and is complementary to the nucleotide sequence that encodes the consensus amino acid sequence A³⁷³HEFGH. The underlined ten base

extensions were added to facilitate subcloning and encode a *SacI* and *SalI* site (in bold) respectively. The third oligonucleotide (CG62) is a 17-base degenerate oligonucleotide and represents the sequences that can encode the N-terminal six residues of the *p*-aminophenylmercuric acetate (APMA)-activated chicken gelatinase as determined by protein microsequencing (Chen et al., 1991). The sequence 5'-TAYAAATTTTCCNMG-3' represents the sequence of the N-terminus Y⁸¹NFFPR.

PCR

PCR was performed as described previously (Lee et al., 1988; Rappolee et al., 1988). Briefly, cDNA was prepared using 3 µg of total RNA from RSV-transformed CEF. Reverse transcription (RT) was carried out in a 20 µl volume of 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 4 mM dNTP, 1 µM 3' PCR primer, and 1 unit/µl M-MuLV reverse transcriptase at 42 °C for 30 min. PCR was performed using the cDNA generated above in 100 µl containing 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.8 mM dNTP, 0.2 µM of each PCR primer and 25 units/ml *Taq* DNA polymerase. A hot start (D'Aquila et al., 1991) was used followed by three low-annealing-temperature cycles of 1 min at 94 °C and 1 min at 55 °C. This was followed immediately by 27 cycles of 1 min at 94 °C, 1 min at 65 °C and 1 min at 72 °C. PCR products were subcloned using the T/A cloning kit (Mead et al., 1991) according to the manufacturer's protocol.

DNA sequencing

DNA was sequenced using the dideoxy-chain-termination method (Sanger et al., 1977) and Sequenase version 2.0 according to the manufacturer's protocol. For sequencing large fragments of DNA a series of nested deletions (Henikoff, 1984) were generated using the Erase-a-base system.

Isolation and characterization of cDNA clones

A 10-day-old chicken embryo λgt11 cDNA library was screened with ³²P-labelled DNA probes as described (Maniatis et al., 1982). Phage plaques (2.5 × 10⁵) were generated in bacterial strain Y1090 after overnight incubation at 37 °C. The DNA from the phage plaques was transferred to nylon membranes and probed with DNA probes as described above for Northern blots. The filters were washed in 0.2 × SSPE/0.1 % SDS at 55 °C and exposed to film overnight. Positive plaques were isolated and purified using standard procedures (Maniatis et al., 1982).

RESULTS

RT-PCR of RSV-transformed CEF RNA yields a 0.95 kb product that encodes a portion of the 72 kDa chicken gelatinase

Members of the MMP family are composed of a series of functional domains. Two of these domains contain the highly conserved sequence motifs of the cysteine-switch (PRCGXPD) and the zinc-binding regions (AAHGXGH), which are involved in the co-ordination of a zinc ion within the proenzymes (Springman et al., 1990; Vallee and Auld, 1990). In order to generate a DNA probe for use in screening a chicken embryo cDNA library, total RNA from RSV-transformed CEF was used as a template in RT-PCR (Rappolee et al., 1988). Two degenerate oligonucleotide primers were designed to hybridize to the nucleotide sequences encoding the conserved motifs mentioned above (see the Materials and methods section). On the basis of

human and mouse proMMP-2 cDNA sequences, the predicted size of the PCR fragment representative of the chicken progelatinase would be 937 bp (Collier et al., 1988; Reponen et al., 1992). A major 0.95 kb PCR product was generated, along with a 0.48 kb and a minor 0.42 kb product (Figure 1a, lane 1).

To determine which of the PCR products represented the chicken progelatinase, Southern blots (Southern, 1975) were performed and probed with ³²P-labelled CG62. This oligonucleotide probe is specific for the nucleotide sequence that encodes the N-terminal six residues of the 62 kDa active chicken gelatinase previously determined (Chen et al., 1991) and which are located between the cysteine-switch and zinc-binding regions. CG62 hybridized to the major band at 0.95 kb, to a 0.48 kb

Figure 1 Ethidium bromide-stained agarose gel electrophoresis and Southern-blot analysis of RSV-transformed CEF RT-PCR product

(a) DNA samples were electrophoresed on a 0.7% agarose gel containing 1 µg/ml ethidium bromide. Lane 1, 10 µl of the RT-PCR reaction; lane 2, T/A clone pTA2-38 digested with *EcoRI* and *HindIII*; lane 3, T/A vector cut with *EcoRI* and *HindIII*; lane 4, gel-purified *EcoRI/HindIII* fragment from pTA2-38. (b) Southern blot of the same gel shown in (a) probed with a ³²P-labelled degenerate oligonucleotide CG62 which hybridizes to the nucleotides encoding the 6 N-terminal residues of the 62 kDa active chicken gelatinase. DNA standards (in kb) are indicated on the left.

Figure 2 Schematic of 72 kDa chicken gelatinase cDNA, cDNA clones and sequencing strategy

A schematic representation of the 72 kDa chicken gelatinase cDNA is shown. The protein-coding region of the cDNA is represented by the box, with the stippled portion representing the signal peptide. The relative positions of the start translation signal (ATG), stop codon (TGA), polyadenylation signal (AATAAA) and poly(A) tail (AAAAA) are indicated. Lines below the box represent the four cDNA clones isolated from the chicken embryo cDNA library and are numbered on the left (size, in kb, indicated in parentheses). The broken line represents the PCR product generated from RSV-transformed CEF RNA that was used as a probe for screening the cDNA library. The arrows above the box represent the nested deletions of clone 3.1 that were used for sequence assembly. The sequence of the 5' end of the cDNA was determined from clone 4.1. The entire DNA sequence was determined from both cDNA strands.

AATTCGGCAAAAGAGAAAAACGGTGCAGAGAGTTAAGATGTGCAGATAAGCAACTAGTGCAGTGTGCAGCCAAAGTAACTGACAGTCAGTCAGAGAAATC -110

TTTAAAGAGGATTGCAAAAATATAGGCAGAATGAAGACTCACAGTGTCTTTGGCTTCTTTTTAAAGTACTATTAAATCCAAGTGTATCTTTTAAACAAA -10
M K T H S V F G F F F K V L L I Q V Y L F N K -4

ACTTTAGCTGCACCGTCACCAATCATTAAAGTTCCTGGAGACAGCACTCCAAAAACAGACAAAGAGCTAGCAGTGAATACCTGAATAAATATTATGGAT +91
T L A A P S P I I K F P G D S T P K T D K E L A V Q Y L N K Y Y G +30
. V A T F . . +30
. V A T F . . +30

GCCCAAAAGACAATTGCAACTTATTGTATTGAAAGATACTTTGAAGAAAATGCAGAAATTTTTGGGCTGCCTGAAACAGGAGATTGGATCAAAACAC +191
C P K D N C N L F V L K D T L K K M Q K F F G L P E T G D L D Q N +63
. . . E S Q +63
. . . E S Q +63

AATTGAGACAATGAAGAAACCCCGCTGTGGTAACCCCGATGTGGCAATTACAACCTCTTTCCAAGAAAGCCAAAATGGGAAAAGAATCATATAACATAC +291
T I E T M K K P R C G M P D V A N Y N F F P R K P K W E K N H I T Y +97
. . . . R D . . . Q . . +97
. . . . R D . . . Q . . +97

AGGATTATAGGCTATACCCCGGATTGGATCCTGAGACAGTAGATGATGCCTTTGCCCGAGCCTTTAAAGTCTGGAGTGTATGTCACGCCACTGAGATTTA +391
R I I G Y T P D L D P E T V D D A F A R A F K V W S D V T P L R F +130
. Q +130
. L +130

ACCGAATAAATGATGGAGAGGCAGACATTATGATTAAATTTGGCCGATGGGAACATGGTGATGGCTATCCATTGATGGCAAAGATGGTCTCTGGCTCA +491
N R I N D G E A D I M I N F G R W E H G D G Y P F D G K D G L L A +163
S . . H +163
S . . H +163

CGCCTTTGCACCGGGGGCAGGAATTGGAGGAGACTCCCATTTTGATGATGATGAAGTGTGGACTCTTGGAGAAGGGCAAGTGGTATAGAGTAAAGTATGGA +591
H A F A P G P G I G G D D S H F D D E L W T L G E G Q V V R V K Y G +197
. T . V +197
. T . V +197

AATGCAGATGGTGAATACTGCAAAATTTCCCTTCTGGTTCAATGGTAAGGAATACAACAGCTGCACAGATGCAGGACGTAATGATGGATTCCCTCTGGTGT +691
N A D G E Y C K F P F W F N G K E Y N S C T D A G R N D G F L W C +230
. L T . . . S . . +230
. L . . . R . . S . . T . . S . . +230

CCACAACCAAGACTTTGATGCAGATGGCAAAATATGGCTTTTGTCCCCATGAGTCACTTTTACAATGGGTGCAATGGTGTATGGACAGCCCTGCAAGTT +791
S T T K D F D A D G K Y G F C P H E S L F T M G G N G D G Q P C K +263
. . . Y N . E K A A E . . . +263
. . . Y N . E K A A +263

TCCCTTTAAATTTCAAGGCCAGTCTTATGACCAGTGTACAACAGAAGGCAGGACAGATGGATACAGATGGTGTGAACCACTGAAGACTATGATAGAGAT +891
F P F K F Q G Q S Y D Q C T T E G R T D G Y R W C G T T E D Y D R D +297
. . . R . . . T . . S +297
. . . R . . . T . . N S +297

AAGAAATACGGATTCTGCCAGAACTGCCATGTCAACAGTTGGTGGAAATTCAGAAGGAGCTCCTTGTGTATTCCCTTCATCTTCCTTGGGAATAAAT +991
K K Y G F C P E T A M S T V G G N S E G A P C V F P F I F L G N K +330
. T +330
. T +330

ACGACTCCTGTACAAGTGCAGGTGCGAATGATGGCAAGCTGTGGTGTGCTTCTACCAGCAGTATGATGATGACCGCAAGTGGGGCTTTTGTCCAGATCA +1091
Y D S C T S A G R N D G K L W C A S T S S Y D D D R K W G F C P D +363
. E S . . . M . . T . A N +363
. E V . . . T . A N +363

AGGATACAGTCTCTTCTTGGTGTGCTGCCCAGCAATTTGGCCATGCGATGGGATTAGAGCACTCCGAGGACCCAGGAGCTCTCATGCCCCGATCTACACC +1191
Q G Y S L F L V A A H E F G H A M G L E H S E D P G A L M A P I Y T +397
. Q +397
. Q +397

TACACCAAGAACTTCCGCCTTTCTCAGGATGACATTAAGGGGATTACAGGAGCTATGAAGTATCCTGATGTGGAACCTTGACCAGGGCCAGGACCAAG +1291
Y T K N F R L S Q D D I K G I Q E L Y E V S P D V E F G F G P G F F +430
. G A . . . I D L - - - - - +424
. H G P . . . A D T D T - - - - - +426

Figure 3 For caption see facing page.

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GGCCAGGACCACGCTCTACCCCTGGACCTGTCACTCCAGAGCTCTGCAAGCAGCAGATTGTATTTGATGGAGTTGCACAAATTAGAGGAGAAATATTTT +1391
G P G P R P T L G P V T P E L C K H D I V F D G V A Q I R G E I F +463
. T . . T . . . . . . . . . . I . . . Q . . . . . I . . . . . . . . . . +457
. T . . T . . . . . . . . . . I . . . Q . . . . . I . . . . . . . . . . +459

CTTCAAAGACAGATTGATGGAGGACTGTAAACCCCTGAGGAAAAACCCACAGGTCCTCTTCTCGTTGCTACATTCTGGCCTGATCTGCCAGAGAAAAATC +1491
F F K D R F M W R T V N P R G K P T G P L L V A T F W P D L P E K I +497
. . . . . I . . . . . T . . . D . . . . . M . . . . . E . . . . . +491
. . . . . I . . . . . T . . . D . . . . . . . . . . . E . . . . . +493

GATGCTGTCTACGAGTCCCTCAGGATGAGAAGGCTGTATTTTTCAGGAAATGAGTACTGGGTTTATACAGCCAGCAACCTGGATAGGGGCTATCCAA +1591
D A V Y E S P Q D E K A V F F A G N E Y W V Y T A S N L D R G Y P +530
. . . . . A . . . E . . . . . . . . . . . I . S . . . T . E . . . . . +524
. . . . . A . . . E . . . . . . . . . . . S . . . T . E . . . . . +526

AGAAACTCACCAGCCTGGGACTACCCCTGTATGTGCAACGCATTGATGCAGCCTTCAACTGGGGCAGAAACAAGAAGACATATATTTTCTCTGGAGACAG +1691
K K L T S L G L P P D V Q R I D A A F N W G R N K K T Y I F S G D +563
. P . . . . . . . . . . . V . . . . . S K . . . . . A . . . . . +557
. P . . . . . . . . . . . Q V . . . . . S K . . . . . A . . . . . +559

ATACTGGAAGTACAATGAAGAAAAAGAAAAATGGAGCTTGAACCCCAAAATTCATTGCGGATTCTTGAATGGAGTTCCAGATAACCTCGATGCTGTC +1791
R Y W K Y N E E K K K M E L A T P K F I A D S W N G V P D N L D A V +597
K F . R . . . V . . . . D P G F . . L . . . A . . A I . . . . . +591
K F . R . . . V . . . . D P G F . . L . . . A . . A I . . . . . +593

CTGGGCTTACTGACAGCGGGTACACCTATTTTTCAAAGACCAGTACTATCTACAAATGAAGACAAGAGTTGAAGATTGTTAAATTTGGCAAGATAA +1891
L G L T D S G Y T Y F F K D Q Y Y L Q M E D K S L K I V K I G K I +630
V D . Q G G . H S . . . . G A . . . . K L . N Q . . . S . . F . S . +624
V D . Q G G . H S . . . . G A . . . . K L . N Q . . . S . . F . S . +626

GTTCTGACTGGTTGGGTTGCTGAACTGTAGAATATATTAATAACCAATATTTACTTTTTTGTATATACCTTATCTGTAATTAGAAATAGATCTGAATG +1991
S S D W L G C * +637
K . . . . . +631
K . . . . . +633

TCAATTACTGGACCAGTCAGGTACTGGCACCCAAACATTAAGTACCAGTACTGTACACATCTGATAATTTAAAAGTGGTTGCTCTCAACATACAAAAG +2091
ATGTTTACGTATCTATTTCTGGTACAATTTTTCAGTTTGTGTAGTCATAGCAATGCTAGTAGCAGAACACGCACGGTTCTTTATCCCTGGTGCTTG +2191
AAGAAAGCATTGAAATCATTGGAGACTGGACCAGACCCCTGTGTAAATCCATCTCAAATAGCCTCAAGTAAGATGTACCAGCAGTGCATTGATTTT +2291
TTTTAATTTTTTTTTTAAATACAACCTCTTTATAAATAGATGGTTGTGTTAATGTTTAAACCTCTTGCGAGTTTGATCATTACTGCCATTATTACA +2391
TAATGATACAAAAGACTGTCAAAGGCACAGTTGCCACGGGATTGATTGTGATGTCATGGAATCATGGCAAGAACTGTATTAAAGTATTGTT +2491
TTAAAGTACTTTCTATTTTAAACCTTAGTTCAACTTAGCAATTTGCTTCTGCACCTTTGTTACTACTCTATAACTTGTCTATACAGACAGAATGACTCA +2591
GATGTTTAAATCAATGTATTATACACAGAAGTCAAGTATTACTGTTTACCTTGTTTATTTTCTGTTCTTATAATATTGAGTTTTTGTTTGGTTAGG +2691
CTTTCGCTTAGGCATTGGGCAACAGAAGGTGTCCTTTCACATATGATGGAATGTTTCTCATTAAAAAAGGTTAAAAAATCCAGAAAAAAT +2791
GTCCCAACATGAAATGCCAATGCTACAAATATAAATTAACACTGTCAAAAAAAAAAAAAACGGTTAAC +2864

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Figure 3 Nucleotide and deduced amino acid sequences of chicken progelatinase cDNA

The first line indicates the nucleotide sequence and the second line shows the deduced amino acid sequence of the chicken gelatinase. The third and fourth lines show the deduced amino acid sequence of human (Collier et al., 1988) and mouse (Reponen et al., 1992) proMMP-2 respectively. Identical residues are indicated by (-), differing residues are given by their one-letter IUPAC lettering. Missing residues are indicated by (-). The numbering of the amino acid residues starts from the first residue of the proenzyme, with the residues of the signal peptide being given negative numbers. The nucleotide sequence is numbered accordingly. The putative initiation of translation (ATG) is marked by (▶▶▶) and the translation termination signal (TGA) is indicated by an asterisk (*). The polyadenylation signal (AATAAA) is double underlined. The additional 18 nucleotides and corresponding six amino acids found in the chicken gelatinase are boxed. The PCR product (excluding the oligonucleotide primer) is underlined. Previously determined (Chen et al., 1991) N-terminal sequences for the chicken proenzyme and active enzyme are contained within ◆◆ and ▶◀ respectively.

product and weakly to other products that were not analysed further (Figure 1b, lane 1).

The PCR products were subcloned into the T/A cloning vector (Mead et al., 1991) and a panel of cloned PCR products was generated. Three of these clones tested positive when probed with CG62 (results not shown). Figure 1(a) (lane 2) shows one clone, pTA2-38, that was digested with *Eco*RI and *Hind*III to generate an insert of 1.0 kb and the vector of 3.0 kb. Lane 3 shows the 3.0 kb T/A vector alone. When probed with CG62, clone pTA2-38 was positive (Figure 1b, lane 2). Clone pTA2-38 was sequenced and the insert was 937 bp in length with an open reading frame that encodes 294 amino acids. This amino acid sequence is 91% and 90% identical with the corresponding regions of human and mouse proMMP-2 respectively. The identity at the nucleotide level is 78% for both human and

mouse proMMP-2 cDNA. The N-terminal sequence of the APMA-activated chicken gelatinase (Chen et al., 1991) that corresponds to CG62 was identified, confirming that the PCR product represents a portion of the 72 kDa chicken progelatinase cDNA.

Cloning of chicken progelatinase from a λ gt11 chicken embryo cDNA library

To determine the entire coding sequence for the chicken progelatinase, the insert from clone pTA2-38 was gel-purified (Figures 1a and 1b, lanes 4) and used to screen a λ gt11 10-day-old chicken embryo cDNA library. Four positive clones, with cDNA inserts that ranged in size from 1.0 to 3.0 kb, were isolated. The cDNAs were subcloned into pBluescript II KS⁺ and

Figure 4 Comparison of deduced amino acid sequences of the 72 kDa chicken progelatinase with human and mouse proMMP-2

The amino acid sequences of chicken progelatinase and mammalian proMMP-2 were aligned as in Figure 3 and the percentage identity was compared using a sliding window of 20 residues. The percentage identity (y -axis) is plotted against the central residue of each 20 residue window (x -axis). The continuous line is chicken progelatinase versus human proMMP-2 and the broken line is mouse versus human proMMP-2. The inserted six amino acids in the chicken gelatinase and the two inserted amino acids in mouse proMMP-2 were excluded to simplify the comparison.

Figure 5 Northern-blot analysis of chicken gelatinase RNA from CEF and RSV-transformed CEF cultured in the absence or presence of PMA

Northern-blot analysis of RNA from CEF (lanes 1 and 2) and RSV-transformed CEF (lanes 3 and 4) cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of PMA. Total RNA (5 μ g/lane) was electrophoresed, transferred to nylon membranes and hybridized with 32 P-labelled pTA2-38 (upper panel). The positions of the rRNAs are indicated on the left. The lower panel is the same Northern blot stained with Methylene Blue to demonstrate RNA loading.

the ends of each cDNA were sequenced. One clone, designated p72K3.1 (Figure 2, clone 3.1), was used to generate a series of nested deletions (Henikoff, 1984). Truncated inserts were partially sequenced and overlapping sequence fragments were assembled as represented in Figure 2. The sequence of the 5' 250 nucleotides were determined from clone 4.1.

The nucleotide and deduced amino acid sequences of the 72 kDa chicken progelatinase are shown in Figure 3. The cDNA is 3072 bp long and translates into a sequence of 683 amino acid residues. A putative ATG start codon, a 26-residue hydrophobic leader sequence and a TGA termination codon are identified in

Table 1 Chicken progelatinase mRNA levels in CEF in response to malignant transformation and/or phorbol ester stimulation

The relative steady-state levels of chicken progelatinase mRNA from eight individual Northern blots probed with pTA2-38 were quantified using scanning densitometry and corrected for total RNA loaded. Expression levels are represented relative to CEF expression which is arbitrarily set as 1.0. Levels are means $\pm$ S.E.M.

Cells	PMA	Expression level
CEF	—	1.0 $\pm$ 0.1
CEF	+	3.0 $\pm$ 0.3
RSV-transformed CEF	—	5.0 $\pm$ 0.3
RSV-transformed CEF	+	6.1 $\pm$ 0.3

the sequence. The deduced amino acid sequence is identical with the previously determined N-terminal sequences of the 72 kDa zymogen and 62 kDa organomercurial-activated enzyme (Chen et al., 1991) (indicated between the symbols $\blacklozenge$ $\blacklozenge$ and $\blacktriangleright$ $\blacktriangleleft$ respectively). The predicted molecular masses for the active enzyme and the proenzyme are 62762 Da and 71690 Da respectively. In addition to the coding sequence, 132 nucleotides of 5' untranslated sequence and 1061 nucleotides of 3' untranslated sequence, including a consensus polyadenylation signal, were found. Three tandem Gly-Pro repeats, were found to be inserted into a proline-rich region of the proenzyme. This insert is represented in Figure 3 between residues Pro⁴²⁵ and Gly⁴³⁰. Mouse MMP-2 has two additional amino acids that are inserted in the proline-rich hinge region of the enzyme (Collier et al., 1988; Reponen et al., 1992). The function of these inserts in the chicken and mouse gelatinases is unknown.

The amino acid sequence identity between the chicken progelatinase and mammalian proMMP-2 is 84%. Interestingly, this level of identity is not evenly distributed throughout the protein sequences. The sequence of the propeptide, gelatin-binding and catalytic domains are more than 88% identical with the sequences of human and mouse proMMP-2. There is more than 90% identity between amino acid residues 1 and 430, which decreases to approx. 75% between amino acids 431 and 637. When the chicken and human proenzyme protein sequences are compared, using overlapping 20-amino-acid-residue windows, the difference between the C-terminal domains of chicken and human progelatinases is more evident (Figure 4). The amino acid sequences of the C-terminal domains of the chicken and mammalian gelatinases become more divergent towards the C-terminus. This divergence is substantial and drops to as low as 45% identity for the 20 amino acids between Val⁵⁹⁰ and Lys⁶¹⁰. However, when human and mouse MMP-2 sequences are compared (broken line, Figure 4), only minimal divergence in the C-terminal domain occurs as the mouse hemopexin domain is 98% identical to the human hemopexin domain.

Northern-blot analysis of RNA from CEF and RSV-transformed CEF shows that steady-state levels of chicken gelatinase mRNA increase upon RSV transformation and PMA stimulation

To determine the transcript size and expression levels of the chicken progelatinase mRNA in normal and transformed CEF, Northern blots were performed on total RNA isolated from CEF and RSV-transformed CEF. The chicken progelatinase is encoded by a 3.3 kb mRNA transcript in CEF cultured in the absence or presence of PMA (lanes 1 and 2, Figure 5). The gelatinase from RSV-transformed CEF cultured in the absence

or presence of PMA is encoded by a 3.3 kb mRNA transcript also (lanes 3 and 4). The Methylene Blue-stained blot is shown to demonstrate equal loading of all samples. The chicken progelatinase mRNA levels increased in response to RSV transformation and/or treatment with PMA. The chicken progelatinase mRNA levels from eight separate experiments were quantified using scanning densitometry and corrected for total RNA loaded (Table 1). RSV transformation results in a 5-fold increase in mRNA levels and PMA stimulation of CEF results in a 3-fold increase in the gelatinase mRNA levels. The effects of PMA treatment and RSV transformation of CEF do not appear to be additive as RSV-transformed CEF cultured in the presence of PMA do not express significantly increased levels of mRNA.

DISCUSSION

The cDNA encoding the 72 kDa chicken progelatinase was cloned and sequenced from CEF. The degree of identity of the chicken progelatinase to 72 kDa human type-IV procollagenase is 85 % at the protein and 75 % at the nucleotide level. The level of identity between chicken and human MMP-2 proteins, however, is not distributed homogeneously over the entire amino acid sequence. These two enzymes are more similar in the N-terminal 430 residues of the molecule (approx. 90 % identity) than in the C-terminal 200 amino acids (less than 70 % identity).

Although the majority of the chicken progelatinase is identical to human MMP-2, the divergence in the C-terminal sequences of the chicken enzyme is structurally interesting and invites speculation on its possible functional significance. The C-terminal domains of MMPs do not appear to be necessary for catalysis as several truncated forms of these enzymes arise spontaneously *in vitro* and they are active (Okada et al., 1988; Clark and Cawston, 1989; Howard et al., 1991). Also, MMPs that lack the C-terminal domain through genetic engineering are catalytically active (Marcy et al., 1991; Murphy et al., 1992a). In addition, native matrilysin lacks the entire C-terminal domain and possesses significant catalytic activity (Quantin et al., 1989; Woessner and Taplin, 1988). However, it has been proposed that the C-terminal domain of MMPs may play a direct role in the more restricted substrate specificities and in the binding of their natural inhibitors, TIMPs (Murphy et al., 1992b; Willenbrock et al., 1993). The C-terminal sequence differences observed in this study between the mammalian and avian gelatinases may help to explain the interactions of these enzymes with their substrates and inhibitors. Chicken gelatinase does cleave the denatured forms of most collagens but, unlike human MMP-2, it does not appear to cleave native type-IV collagen (Chen et al., 1991). In addition, mammalian MMP-2 is normally isolated in a non-covalent, but high-affinity complex with the 21 kDa member of the TIMP family, TIMP-2 (Goldberg et al., 1989; Stetler-Stevenson et al., 1989). Although chicken progelatinase was originally reported (Chen et al., 1991) to co-purify with a similar protein, which is probably a member of the recently described family of chicken inhibitors of metalloproteinase (ChIMPs) (Pavloff et al., 1992), highly transformed chicken fibroblasts secrete a large amount of progelatinase in a TIMP-free form. The appearance of naturally occurring TIMP-free proMMP-2 may reflect a reduced affinity of chicken progelatinase for TIMP-2, possibly affected by the divergent C-terminal domain. These observed differences in both substrate specificity and TIMP-2 interaction might be addressed experimentally by the construction of genetically engineered human-chicken chimeric molecules and such studies are now ongoing.

The secretion of this enzyme by CEF is elevated upon transformation by RSV (Chen et al., 1991). The steady-state level of mRNA is elevated also upon RSV transformation. Upregulation of the progelatinase mRNA can be achieved by culturing CEF in the presence of the phorbol ester, PMA. Unlike human MMP-2, which appears to be relatively resistant to PMA (Huhtala et al., 1990; Mackay et al., 1992), the chicken progelatinase responds positively to PMA treatment, although not to the same extent as other mammalian MMPs such as MMP-1 and MMP-3 (Angel et al., 1987; Mackay et al., 1992). A positive response of human MMP-2 to PMA and transforming growth factor β has been reported (Overall et al., 1991); however, there is only a 2-fold induction of mRNA and protein levels. These results suggest that the regulation of the chicken progelatinase may be different from that of mammalian proMMP-2, which can be downregulated but is resistant to external positive stimuli (Tryggvason et al., 1990; Huhtala et al., 1991). The chicken progelatinase appears to be more similar to mammalian MMP-3 and MMP-1 in terms of its response to external stimuli and viral transformation. Additional studies on the 5' regulatory elements within the chicken progelatinase promoter are under investigation.

The increased production of MMPs has been implicated in various pathological processes such as rheumatoid arthritis and cancer metastasis (Docherty et al., 1992). Degradation of ECM components is an important step in the progression of many of these diseases. RSV-transformed CEF are known to extensively degrade the ECM that is produced by normal fibroblasts (Fairbairn et al., 1985; Quigley et al., 1987). This invasive phenotype is believed to be mediated by urokinase-type plasminogen activator and one or more MMPs (Sullivan and Quigley, 1986; Quigley et al., 1990). The chicken gelatinase is elevated at both the RNA and protein levels upon oncogenic transformation, as well as by stimulation with phorbol ester. These results in conjunction with previous studies (Chen et al., 1991), suggest that the upregulation of chicken progelatinase, as a result of RSV transformation, may be partially responsible for the high levels of pericellular proteolysis that has been observed with these cells.

This study represents the first avian MMP to be cloned and sequenced. Because of the evolutionary distance between avians and mammals, the reported sequence similarities and differences may help define specific sequences involved in substrate specificity, interactions with TIMP, zymogen-activation properties and gene regulation. The RT-PCR technique that was utilized in this study is a powerful method for cloning proteinases from various species, as shown for serine- and cysteine-proteinases from a protozoan parasite (Sakanari et al., 1989), a mouse macrophage elastase (Shapiro et al., 1992) and rat proMMP-2 (Marti et al., 1993). In the present study, the PCR primers were constructed on the basis of highly conserved sequence motifs that flank unique sequences specific to individual enzymes and species. As the PCR primers are designed on the basis of gene-family-specific sequences, this approach can be used where cloning has been hampered by the lack of gene- or protein-specific sequence data.

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