Cloning of rat 92-kDa type IV collagenase and expression of an active recombinant catalytic domain

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Abstract A full-length cDNA for rat 92-kDa type IV collagenase was isolated and sequenced. RNase protection assay revealed tissue specific differential expression of the 92-kDa type IV collagenase in the rat during development. Natural and modified forms of the 92-kDa type IV collagenase were expressed. One active protein, 92-CD, contained only the putative catalytic domain. Large quantities of the 92-CD were expressed in *Escherichia coli*, extracted from inclusion bodies, purified, and refolded to an active form. This recombinant protein was able to cleave denatured and native collagen and was inactivated by known MMP inhibitors.

Key words: Metalloproteinase; MMP-9; Eukaryotic expression; Prokaryotic expression; Refolding

1. Introduction

The extracellular matrix (ECM) provides structural support and assists in cell proliferation, adhesion, migration, and tissue morphogenesis. The mammalian matrix metalloproteinases (MMPs) degrade ECM in physiological and pathological processes involving extensive ECM remodeling (reviewed in [1]). The MMPs are structurally homologous and evolutionarily related molecules composed of defined functional domains [2,3]. After cleavage of a signal peptide domain of about 20 amino acids, the MMPs are secreted in latent form. Upon activation, the N-terminal propeptide domain is cleaved to leave the active form of the MMP [4]. With the exception of matrilysin, the active MMPs have a common domain structure consisting of a catalytic domain, a linker peptide, and a C-terminal domain with sequence similarity to hemopexin and to the ECM component vitronectin; matrilysin lacks the C-terminal domain. The C-terminal domain is essential for complexing the proenzyme with tissue inhibitor of metalloproteinase (TIMP) [5]. The activity of the MMPs can be inhibited by endogenous inhibitors like α-2 macroglobulin and tissue inhibitors of metalloproteinases as well as by rapid autodegradation of the C-terminal domain.

Like other members of the MMP family, the 72-kDa and 92-kDa type IV collagenases [gelatinase A (MMP-2); gelatinase B (MMP-9)] contain the basic structure of propeptide, catalytic, and hemopexin domains [6–8]. They differ from other MMPs by the presence of three 58–59-amino acid residue internal repeats that are homologous to the collagen-binding domains of fibronectin, and the 92-kDa type IV collagenase has an additional domain that is similar to type V collagen [6,9]. They are located on a different chromosome than other members of the MMP family [3]. The type IV collagenases are of special interest because of their degradative activity against basement membrane type IV collagen and their expression in cells of emigrational or invasive nature during physiological and pathological processes [10–14]. The mechanisms by which type IV collagenases contribute to physiological and pathological processes remain incompletely understood. The aim of the present study was to clone the rat 92-kDa type IV collagenase cDNA, express and purify an active and stable modified recombinant protein in *E. coli*, to allow better characterization of its in vitro and in vivo properties. The removal of the propeptide obviated the need for proteolytic or organomercurial activation, and the removal of the C-terminal fragment eliminated autolytic sites and made the recombinant protein resistant to autodegradation ideal for experimental usage.

2. Materials and methods

2.1. Cloning rat 92-kDa type IV collagenase

A 262 bp probe (bp 371–633 of the mouse clone) was cloned by RT-PCR with primers selected from conserved regions between mouse and human (Genbank accession nos.: x72795 and J05070) and used to screen a rat macrophage cDNA library [15]. Isolated phage plaques were subjected to in vivo excision for recombinant pBluescript phagemid. After sequencing, it was found that the cloned cDNA lacked 375 bp at the 5′ end of the mRNA of rat 92-kDa type IV collagenase (clone pM92-1.9). To obtain the 5′ end cDNA, RT-PCR was performed with a degenerate upstream primer based on homologies with the 5′ end of the human and mouse sequence, 5′-GCTCTAGAATGAG(TC)CCCTGGCAGCCCCTG-3′, and the downstream primer, 5′-CTCAAGTGGCACCATCATAACATC-3′, according to the 5′ end sequence of pRat92-3. RNA was isolated from fetal kidney and was reverse transcribed.

2.2. Tissue-specific expression of 72-kDa and 92-kDa type IV collagenase

5 μg of total RNA from fetal and adult rat organs was studied by RNase protection assay [16]. For the rat 92-kDa type IV collagenase, a 375 nucleotide riboprobe was transcribed from the PCR-cloned cDNA (Fig. 1). For the rat 72-kDa type IV collagenase, a cDNA clone was obtained from Dr. David Lovett (Veterans Affairs Medical Center, San Francisco, CA) to generate a 222 nucleotide riboprobe. A third probe, the 114 nucleotide riboprobe of the glyceraldehyde-3-phosphate dehydrogenase (GAP), was used to normalize the amounts of RNA in different lanes.

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Abbreviations: 72-kDa type IV collagenase, gelatinase A; 92-kDa type IV collagenase, gelatinase B; 92-CD, catalytic domains; t92-CD, truncated 92-CD; bp, base pairs; cDNA, DNA complementary to RNA; GAP, glyceraldehyde-3-phosphate dehydrogenase; MMP, mammalian matrix metalloproteinase(s); mRNA, messenger RNA; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RNA, ribonucleic acid; RNase, ribonuclease; RT, reverse transcription; SDS, sodium dodecyl sulfate; TIMP(s), tissue inhibitor(s) of metalloproteinase(s)

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2.3. Expression and characterization of rat 92-kDa type IV collagenase and its catalytic domain (92-CD)

For the expression of 92-CD, a fragment coding for amino acid residues 107–463 (CD-92) was amplified by PCR, ligated to the leader sequence of KC cDNA [17], and subcloned into pCAGGS to obtain pCAGGS92-CD. The recombinant DNAs were transfected into subconfluent COS-7 by electroporation of 8×10^6/ml cells at 1500 V, 1000 μF [15]. The antisense clone was used as a control for mock transfection. After 72 h incubation, the supernatant was collected for Western blot and zymography. For expression of 92-CD in *E. coli*, the same PCR fragment was cloned into pETM1 [17]. To obtain a truncated 92-CD without the zinc-binding site (t92-CD), a fragment corresponding to amino acid 107–409 (t92-CD) was amplified by PCR and cloned into pETM1. The recombinant plasmids, pET92-CD and pETt92-CD, were transformed into BL21(DE3). After induction, the inclusion bodies were extracted with a buffer containing 6 M urea and loaded on a Ni-NTA affinity column (Qiagen, Chatsworth, CA). The purification procedure was carried out as described previously [18]. To refold the protein on column, refolding buffer containing 5 mM $CaCl_2$/20 mM Tris/0.2 M NaCl with urea gradient of 4 M–0.5 M was added at a rate of 0.5 ml/min. After refolding, the protein was eluted with 80 mM imidazole/5 mM $CaCl_2$/20 mM Tris/0.2 M NaCl/0.5 M urea and then dialyzed against phosphate-buffered saline (PBS). For zymography, the method described by Hibbs et al. [19] was used.

The 92-CD was expressed in *E.coli* and used to immunize a rabbit as described previously [17]. Western blot was carried out as described previously [15]. Collagenase activity was studied using 10 μg of rat tail type I collagen (Boehringer Mannheim, Indianapolis, IN) incubated with different amounts of recombinant protein and enzyme inhibitors for 4 h at 37°C. The reactions were stopped by adding SDS sample buffer containing β-ME, boiled and loaded for SDS-PAGE. The protein bands were visualized with Coomassie Blue staining.

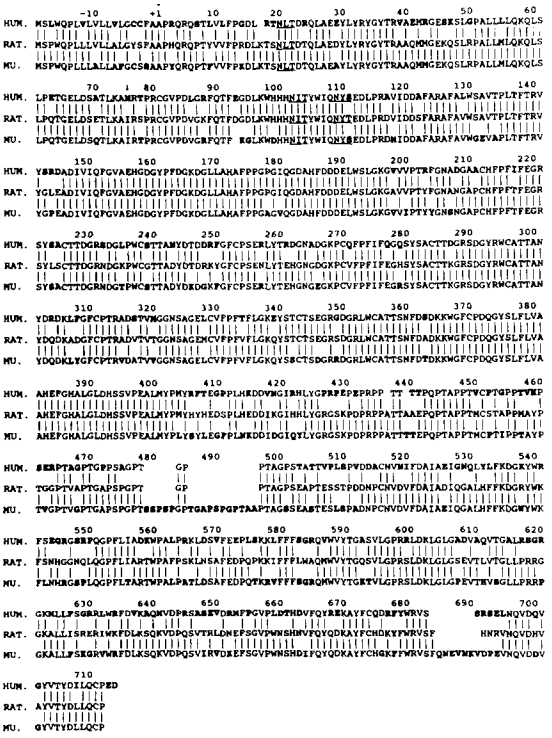

Fig. 1. Comparison of amino acid sequences of human, rat, and mouse 92-kDa type IV collagenase deduced from cDNA sequences. Accession number of the rat 92-kDa type IV collagenase, U36476. Numbering of the amino acids is shown beginning with the first residue of the propeptide; residues of the signal peptide have negative numbers. Residues of identity are linked with bars. The conserved and putative attachment sites for asparagine-linked oligosaccharides are underlined.

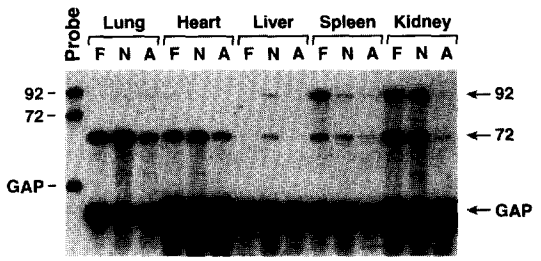

Fig. 2. 92-kDa type IV collagenase (92) and 72-kDa type IV collagenase (72) expression in fetal (F), newborn (N), and adult (A) rat tissues assessed by RNase protection assay.

3. Results and discussion

3.1. Cloning of rat 92-kDa type IV collagenase

Four positive clones, each being 1.9 kb in size, were isolated. Sequence analysis revealed that these four clones were identical and all were partial cDNA clones containing only the 1752 bp coding sequence (nt 375–2127). Further screening of the same cDNA library did not yield any clone with additional 5′ sequences, and polymerase chain reaction (PCR) using the 5′ end primer confirmed that this library does not contain full-length cDNA clones for rat 92-kDa type IV collagenase. Similar difficulties have been encountered by other groups in cloning the cDNA of three other MMPs — human and rat 72-kDa type IV collagenase (gelatinase A) [7,9,20] and mouse 92-kDa type IV collagenase [8] — a problem likely to have arisen from site-specific premature termination of first-strand synthesis during cDNA library construction. To construct the full-length cDNA, a primer containing the conserved 5′-end sequence between human and mouse 92-kDa type IV collagenase was used in RT-PCR of fetal rat kidney total RNA. A 375 bp RT-PCR band was obtained and pieced together with the 1.9 kb partial cDNA. This full-length cDNA was completely sequenced, and Fig. 1 shows the amino acid sequences deduced from the cDNA sequence (Accession #U36476). Comparison of amino acid sequences of the rat 92-kDa type IV collagenase to that of the human and mouse shows a 74% and 80% homology, respectively. Interestingly, the rat enzyme lacks the two extra stretches of amino acids present in the type V collagen and the hemopexin domains of the murine enzyme (Fig. 1). In addition to the coding region, the cDNA also contains a 217 bp 3′UTR including the translation stop codon TGA and the polyadenylation signal (data not shown).

3.2. Tissue-specific expression of mRNA for 72-kDa and 92-kDa type IV collagenase

RNase protection assay was used to determine and compare the mRNA expression of the 72-kDa and 92-kDa type IV collagenases in various tissues (Fig. 2). As can be seen, expression of both enzymes is tissue specific and differentially regulated during development. The 92-kDa type IV collagenase expression was more restricted, compared with that of the 72-kDa type IV collagenase. In the fetal and newborn kidney as well as in fetal spleen, the 92-kDa type IV collagenase is actively expressed, suggesting its role in the development of rat kidney and spleen.

3.3. Expression and characterization of rat 92-kDa type IV collagenase and its catalytic domain (92-CD)

Full-length human 92-kDa type IV collagenase as well as a number of other full-length MMPs have been expressed in and purified from *E. coli* [21–23]. While all these full-length MMPs are active after organomercurial or proteolytic activation, they quickly degenerate due to autolysis of the C-terminal domains. To circumvent this problem, the cDNA was modified and the recombinant protein expressed to obtain an abundant source of active and stable enzyme. Matrilysin (PUMP; MMP-7) naturally lacks the C-terminal hemopexin-like domain and is an active enzyme [24]. Stromelysin and 72-kDa type IV collagenase are active with this domain removed [25–27]. On the other hand, fibroblast and neutrophil collagenases lacking the C-terminal domain lose the degradative activity against collagens [28,29]. To investigate the effect of C-terminal domain deletion on the 92-kDa type IV gelatinase, the rat cDNA was subcloned into various vectors and expressed in different hosts. To minimize other complicating factors like glycosylation and protein folding, the full-length and truncated rat 92-kDa type IV collagenase were expressed in mammalian cells. This was done by subcloning the full-length and truncated fragments of rat 92-kDa type IV collagenase cDNA into mammalian expression vector pCAGGS and transfecting different constructs into COS-7 cells. Both the full-length rat 92-kDa type IV collagenase and its catalytic domain (92-CD) expressed in COS-7 were in agreement with the calculated molecular mass as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gelatinolytic zymogram, and Western blot (Fig. 3). In the supernatant of full-length 92-kDa type IV collagenase transfectant, an extra band appeared, indicating that it is secreted and processed (Fig. 3).

Two recombinant forms, 92-CD (coding for amino acid residues 107–463) and truncated 92-CD (t92-CD, 107–409),

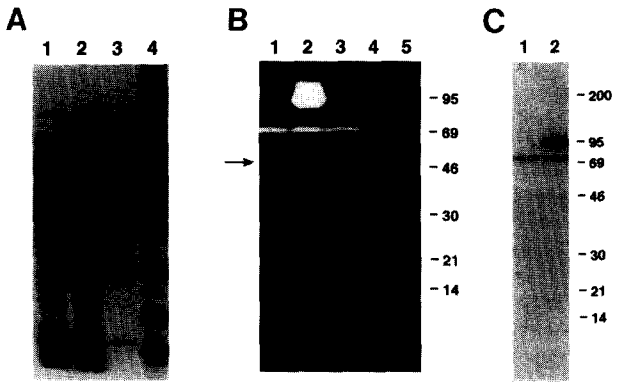

Fig. 3. Expression of rat 92-kDa type IV collagenase and/or its derivatives in various host cells. (A) SDS-PAGE analysis of the expression of 92-CD in *E. coli*. Lane 1, non-induced; lane 2, induced with IPTG for 2 h; lane 3, affinity-purified 92-CD; lane 4, rainbow marker (Amersham, Arlington Heights, IL). (B) Gelatin zymogram analyses of the recombinant proteins. Lane 1, 92-kDa type IV collagen antisense in COS-7; lane 2, full-length sense 92-kDa type IV collagenase in COS-7; lane 3, 92-CD in COS-7; lane 4, 92-CD in *E. coli*; lane 5, t92-CD. The arrow denotes processed 92-kDa type IV collagenase. (C) Western blot analysis of full-length 92-kDa type IV collagenase expressed in COS-7 cells. Western immunoblotting was carried out using a rabbit polyclonal antibody raised against the 92-CD expressed in *E. coli*. Lane 1, antisense, and lane 2, sense, from the transfectant supernatant of COS-7 cells.

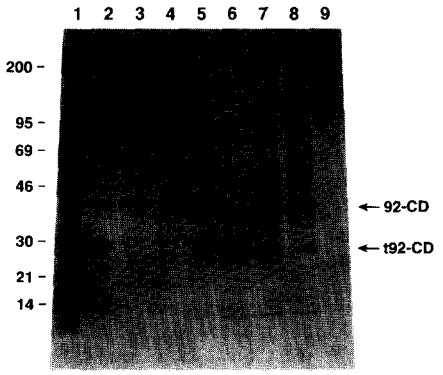

Fig. 4. SDS-PAGE analysis of collagenase activity of 92-CD and t92-CD is shown. Lane 1 is the rainbow marker. 10 μg of rat type I collagen was incubated with t92-CD or 92-CD in the presence of the inhibitors; t92-CD had no effect on collagen digestion (lane 2). Dose-dependent digestion of collagen by recombinant 92-CD at 10 ng, 20 ng, 100 ng, and 200 ng is shown (lane 3 to lane 6). 1 mM of EDTA and 5 mM of phenanthroline were shown to inhibit the collagen digestion of 200 ng 92-CD (lanes 7 and 8). Collagen alone without enzyme is shown in lane 9.

of rat 92-kDa type IV collagenase were expressed in *E. coli*. The t92-CD was without the zinc-binding domain and served as a negative control. The purified 92-CD and t92-CD were expressed as a single band at 38-kDa and 29-kDa, respectively, in agreement with the calculated molecular weight (Fig. 4). The recombinant 92-CD and t92-CD were present in inclusion bodies. The 92-CD was extracted from the inclusion bodies and was functionally renatured with a high yield (40 mg/liter) following the method previously described [17]. The refolded 92-CD was fully active, sensitive to inhibitors, and very stable (Fig. 4).

Type IV collagenases have been shown to be important proteinases in tissue remodeling in many physiological and pathological processes. The high level and functional expression of the catalytic domain of 92-kDa type IV in *E. coli* provides a recombinant protein suitable for structure determination by nuclear magnetic resonance spectroscopy and X-ray crystallography, as well as for mechanistic studies of catalysis and inhibition. Availability of the rat probe and antibody will be useful in studying pathological and physiological functions of this enzyme in rat model systems for human diseases.

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