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A 92 kDa GELATINASE (MMP-9) CLEAVAGE SITE IN NATIVE TYPE V COLLAGEN

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Native type V collagen molecules resist mammalian collagenase but are cleaved by certain gelatinases. We report a prominent site of cleavage within the collagen type V molecules by 92 kDa gelatinase (MMP-9). The enzyme was purified from conditioned medium of a rabbit synovial cell line (HIG-82). It cleaved native type V collagen from bovine bone in solution at two molecular sites, one near the amino-terminus, the other producing a 3/5 C-terminal fragment. Amino-terminal sequence analysis of the individual α chains from this latter fragment showed that MMP-9 had cleaved between residues Gly439-Val in both α1(V) and α(XI) and between residues Gly445-Leu in the α2(V) chain. These sites are close to the previously reported trypsin-cleavage site. The findings imply that gelatinases may be necessary for initiating or completing degradation of type I/type V copolymeric fibrils for growth and remodeling of extracellular collagen.

Type V collagen is widely distributed in relatively minor amounts in all connective tissues that contain type I collagen (for a review see ref. 1). It belongs to the class of fibril-forming molecules that includes types I, II, III and XI collagens, all characterized by a long, uninterrupted triple-helical domain (2). The fibrils and individual native collagen molecules are usually highly resistant to degradation by most proteases. Types I, II and

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III collagens are specifically cleaved by mammalian collagenase primarily at a single locus (Gly-Ile or Gly-Leu bond) which generates 3/4 and 1/4 length fragments (3). Types V and XI collagens, however, resist mammalian collagenase cleavage (4,5), but thrombin, plasmin and trypsin will cleave native type V collagen (6,7) as will metalloproteases with gelatinolytic activity (8-15). The 92 kDa gelatinase, also known as matrix metalloproteinase 9 (MMP-9) (16), degrades native types IV and V collagens into discrete fragments, and has been referred to either as a type IV collagenase (13-15,17) or as a type V collagenase (10-12). As an additional twist, this enzyme molecule itself was reported to have a region with amino-acid sequence homology to a helical sequence in the α2(V) collagen chain (17). The site(s) of attack within the type V collagen molecule have, however, not been determined. We report here the results of microsequence analysis of fragments produced from type V collagen by a 92 kDa gelatinase purified from a synovial-cell conditioned medium.

MATERIALS AND METHODS

Enzyme Purification

A rabbit synovial cell line (HIG-82), previously established (18), was kindly supplied by Dr. C.H. Evans, was the source of the 92 kDa gelatinase. The cell line was grown to confluency and was then transferred to a serumless media containing 100ng/ml of phorbol myristate acetate (PMA) . After 3 days of incubation at 37°C the cells were centrifuged, the recovered Medium was concentrated 10X and then dialyzed against 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.6. The retentate was fractionated on a DEAE-3SW HPLC column (Beckman), eluting with a linear gradient of 0-0.4M NaCl in 0-0.5 M Tris-HCl, 10 mM $CaCl_2$, pH 7.6 at 1 ml/min over 30 min.

Protease Assays

Gelatinolytic activity was determined by zymography on SDS-PAGE (19). Briefly, gelatin (2 mg/ml final concentration) was included in 7.5% acrylamide SDS-gels and aliquots of the fractions spanning the HPLC chromatogram were run without heat-denaturing the sample. After electrophoresis, the gels were washed in buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM $CaCl_2$, pH 7.6, containing 2% Triton X-100 (v/v), then incubated in the same buffer containing 0.02mM of 4-aminophenylmercuric acetate (APMA, final concentration) for 16h at 37°C. After incubation, gels were rinsed in distilled water and stained in Coomassie brilliant blue and destained in 10% methanol, 10% acetic acid in water (v/v). The gelatinolytic activity was indicated by clear bands on a blue background. Collagenase activity was determined by incubating type I collagen with aliquots of the HPLC fractions and caseinase activity by degradation of [^{14}C] casein.

Collagen Degradation Products

Fractions from the DEAE-ion exchange column showing gelatinolytic activity on substrate SDS-PAGE were pooled for degradation studies on native collagen in solution. Pepsin-solubilized types II and XI collagens purified from cartilage and type V

collagen purified from bone were incubated with the enzyme preparation at 30° or 32°C for 16h. At the end of the incubation, 5mM EDTA (final concentration) was added to terminate the enzyme activity. The degradation products were analyzed by SDS-PAGE (7.5% acrylamide).

Amino-terminal Sequence Analysis

The resolved cleavage products from native type V collagen were transblotted onto PVDF (polyvinylidene difluoride) membrane and subjected to amino-terminal sequence analysis on a Porton model 2090E protein microsequencer using the manufacturer's standard program.

RESULTS

The 92 kDa gelatinase was resolved from collagenase and caseinase activities in a single step by the DEAE-ion exchange HPLC. Figure 1 shows the elution profile of the proteins in the medium conditioned by the rabbit synovial cells. Fractions spanning the chromatogram were assayed for gelatinolytic activity using gelatin-SDS-PAGE (Fig. 1 inset). Those fractions showing the main gelatinolytic activity are shown. The clear zones in the gel indicated the protein bands with gelatinase activity. The major activity was seen at 92 kDa as judged by molecular weight markers. Stromelysin, determined by caseinase activity, and collagenase determined by degradation of type I collagen at 25°C were not retained by the column (data not shown).

The purified enzyme degraded types V and XI collagens, but not type II collagen, at 32°C primarily to small fragments that ran in the buffer front (Figure 2a), consistent with the reported action of gelatinases on types V and XI collagens (10-15). To determine whether there were preferred sites of attack within the native type V collagen molecule, the reaction temperature was lowered. At 30°C, larger fragments were evident which indicated that the enzyme had cleaved native type V collagen molecules at two

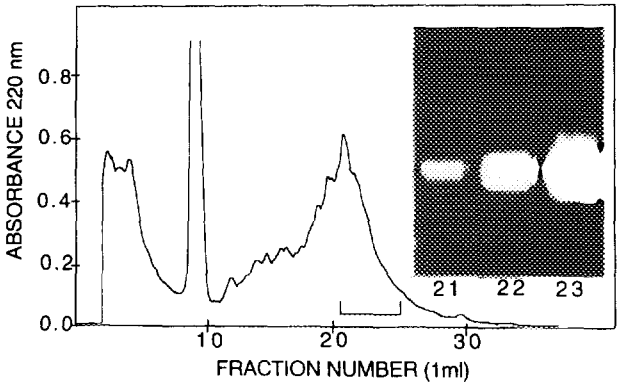

FIGURE 1. Anion-exchange HPLC of the proteins in the medium conditioned by the rabbit synovial cell line. The elution position of the 92 kDa gelatinase is indicated and shown on gelatin-SDS-PAGE fractions 21-23 (inset).

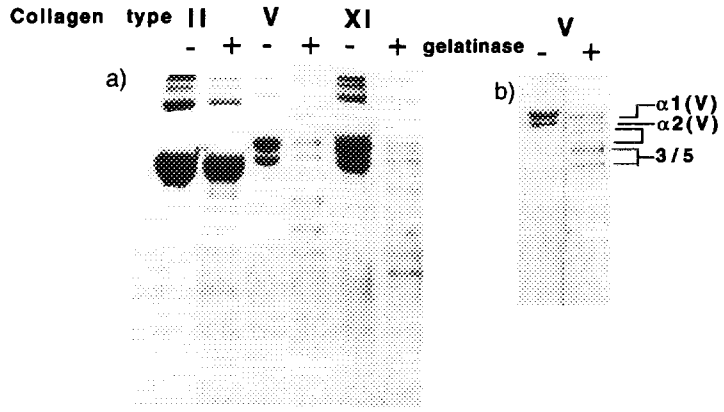

FIGURE 2. SDS-PAGE of digests of collagens with the 92 kDa gelatinase.
(a) incubation at 32°
(b) incubation at 30°C
- = without gelatinase
+ = with gelatinase

sites (Figure 2b). Their identity showed their location was in the amino-terminal half of the molecule. Thus, the major fragments had apparent molecular weights of about 60 kDa (Figure 2b, 3/5 components). On amino-terminal sequence analysis, the upper 3/5 band gave two sequences, one matched the α1(V) chain beginning at Valine440; the other matched the α1(XI) chain beginning at Valine440 (Figure 3). The lower 3/5 band gave one amino-terminal sequence that matched the α2(V) chain beginning at Leucine446 (Figure 3). The results indicate that all three chain components of the type V collagen (20), α1(V), α2(V) and α1(XI), were cleaved by the gelatinase as native heterotrimeric molecules to generate a 3/5 molecular fragment that retained a stable triple-helix at 30°C. Bands of higher molecular weight were also evident in the 30°C digest, which on longer digestion grew fainter as the 3/5 components intensified. The

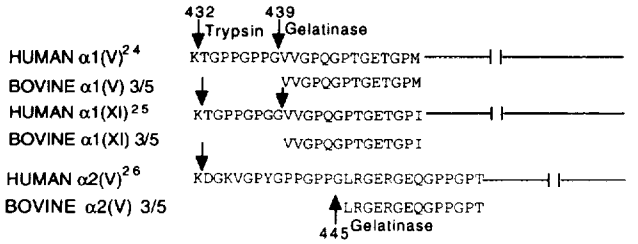

FIGURE 3. Amino-terminal sequences of gelatinase-derived fragments of type V collagen. Matching sequences from reported cDNAS (24-26) are shown for comparison. The location of the trypsin cleavage sites in type V collagen chains identified previously are also shown (data from ref. 7).

sites of gelatinase cleavage in type V collagen identified here are near the trypsin sites identified previously in type V collagen chains (7, Figure 3).

DISCUSSION

Previous studies have shown that types V and XI collagens resist mammalian collagenase digestion, apparently because they lack the susceptible 3/4 cleavage site required by the enzyme (4,5). Whether alternative proteases have evolved for specifically degradating types V and XI collagens *in vivo* has not been clearly defined. Metalloproteinases with gelatinase activity are suspected to be active against these native collagens (8-15).

The present results establish that the native type V collagen molecule contains at least two sites in the triple-helix that are cleaved by 92 kDa gelatinase (MMP-9) at about 1/4 and 2/5 of the molecular length from the N-terminus. This is consistent with the observations of Mainardi et al. who concluded there is more than one gelatinase cleavage site in the native type V collagen molecule (10). In contrast with the primary cleavage site for collagenase (MMP-1) near the C-terminus in types I, II and III collagens, the findings show that the primary site of attack in type V collagen is near the amino-terminus. Interestingly, trypsin also can cleave the native type V collagen molecule very close to the gelatinase cleavage site (7). This microdomain apparently is a region of reduced helical stability. Similarly, for example, type III collagen is also selectively cleaved by trypsin and elastase close to the collagenase cleavage site, indicating a weak helical domain (21,22).

There is mounting evidence that type V collagen exists in the matrix as a copolymer with type I collagen, and that the retained type V N-propeptides may regulate the hybrid fibril diameter (23). With the above considerations, this implies that proteases specific to type V molecules may be involved in the degradation of fibrils necessary for tissue growth and remodeling. The 92 kDa gelatinase, and perhaps other gelatinases, may be performing the dual role of initial attack on native type V and XI collagens and degrading denatured fragments of types I, II and III collagens released by mammalian collagenase attack.

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