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Mercurial activation of human PMN leucocyte type IV procollagenase (gelatinase)

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Autoproteolytic activation and processing of human polymorphonuclear leucocyte (PMNL) type IV procollagenase (gelatinase) was initiated by $HgCl_2$ and was investigated by kinetic analysis and N-terminal sequence determination of the reaction products. In the first instance the propeptide domain was lost by subsequent cleavage of the Asp^{15}–Leu^{16}, Glu^{40}–Met^{41}, Leu^{52}–Leu^{53} and Ala^{74}–Met^{75} peptide bonds. The PRCGVPD sequence motif (residues Pro^{78}–Asp^{84}), which is conserved in all metalloproteinases and expected to be relevant for latency, remained uncleaved at the activated enzyme. The generated intermediate was further processed by three C-terminal cleavages. The Glu^{666}–Leu^{667}, Ala^{506}–Glu^{507} and Ala^{398}–Leu^{399} bonds were hydrolysed sucessively. From the fragmentation products we were able to conclude that three released fragment peptid es contained unpaired free cysteine with the residues Cys^{497}, Cys^{653}, Cys^{683}. Cleavage of the first C-terminal peptide bond resulted in the loss of one of the conserved Cys residues of the hemopexin-like domain, whereas the Cys residue of the PRCGVPD motif was retained at the fully active enzyme. The possibility of an entirely different activation mechanism for PMNL type IV procollagenase is discussed.

Type IV collagenase; Neutrophil; Mercurial activation

1. INTRODUCTION

Type IV collagenases (gelatinases) play key roles in matrix remodelling and basement membrane degradation and contribute to the metastatic potential of tumor cells [1]. The two type IV collagenases are unique members of the matrix metalloproteinase family with regard to their domain structure. The M_r 72,000 and the M_r 98,000 type IV collagenase share a fibronectin-like domain, which is inserted 175 residues preceeding the proposed zinc binding motif. This domain is homologous to the collagen binding motif of fibronectin and responsible for gelatin binding of the proenzymes [2]. The M_r 98,000 type IV collagenase contains an additional domain with sequence similarities to the α_2 chain of type V collagen [3]. The function of this insertion behind the zinc binding motif has still to be determined.

The mode of activation of metalloproteinases in vivo is unknown. Activation in vitro was the target of intensive studies during the last ten years [3–9]. Exposure of the purified proenzymes to proteinases, organomercurials, oxygen radicals or chaotropic agents led to the generation of enzymatic activity.

Abbreviations: PMNL, polymorphonuclear leucocytes; RP-HPLC, reversed-phase high-performance liquid chromatography; TBST, Tris-buffered saline Tween; TIMP-1, tissue inhibitor of metalloproteinases-1.

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As sequence data became available different groups developed theoretical activation mechanisms to explain the experimental data [10,11]. A stable complex between the conserved Cys residue of the PRCGVPD sequence motif within the propeptide domain and the active site zinc might be the basis for latency. It is believed that all modes of activation require the removal of this Cys residue out of the coordination sphere of the zinc binding centre as the most important step. This has recently been supported by site directed mutagenesis experiments with transin, the rat homologues of human stromelysin [12,13]. Mercurial compounds as well as oxygen radicals are assumed to activate by modifying the chelating cysteine sulfhydryl group. Latent metalloproteinases are activated by human neutrophils by an oxidative process in vivo [14].

In this paper we present the first data for the individual steps in the activation process of PMNL type IV procollagenase induced by $HgCl_2$ and discuss a modified activation mechanism for this enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Dinitrophenyl-peptide was purchased from Bachem (Bubendorf, Switzerland). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. *Purification of PMNL type IV procollagenase, activation and enzyme assay*

PMNL type IV procollagenase was purified as recently published [15]. Proenzyme activation was achieved by incubation with $HgCl_2$ at 37°C. Type IV collagenase activity was determined by proteolytic

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degradation of the synthetic octapeptide (dinitrophenyl-Pro-Gln-Ile-Ala-Gly-Gln-D-Arg-OH) as described by Masui [16].

2.3. *Protein determination*

Protein concentrations were estimated from A_{280} assuming an absorption coefficient of 1.0 $M^{-1} \cdot cm^{-1}$.

2.4. *Kinetic analysis of $HgCl_2$-induced activation of PMNL type IV procollagenase and separation of the reaction products by RP-HPLC*

PMNL type IV procollagenase was activated for between 5 min to 2 h at 37°C using 1 mM $HgCl_2$. The reaction mixture was directly subjected to a Bakerbond wide pore C_{18}-column (4.9 × 250 mm). The separations of the reaction products were performed at a constant flow rate of 0.8 ml/min using a linear gradient from 0–60% acetonitrile. Peptides and proteins were detected at 214 nm, collected, lyophilised and subjected to automated amino acid sequence determination using a microsequencer (Model 810, Knauer, Berlin, Germany).

2.5. *SDS/PAGE*

SDS/PAGE was performed according to the method of Laemmli [17]. The proteins were visualised by silver staining [18].

3. RESULTS

3.1. *Autoproteolytic activation and processing of human type IV procollagenase induced by $HgCl_2$*

Type IV procollagenase was purified from human PMN leucocytes. The N-terminus of the enzyme was found to be identical to that of the proenzyme from SV40-transformed lung fibroblasts [3,15]. As shown by SDS/PAGE (Fig. 1, lane 2), PMNL type IV procollagenase consists of three forms with different molecular masses (M_r 220,000, 125,000 and 98,000). Under reducing conditions these forms result in M_r 98,000 and M_r 28,000 subunits [15]. The isolated latent enzyme showed no activity against the synthetic DNP-octapeptide.

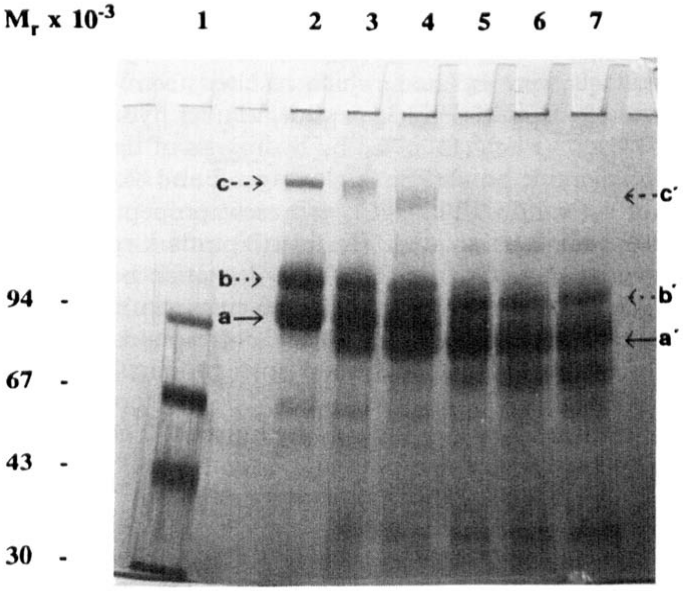

Fig. 1. Time-dependent activation of PMNL type IV procollagenase by $HgCl_2$: SDS/PAGE analysis of generated products. (Lane 1) molecular mass marker; (lane 2) starting material, PMNL type IV procollagenase; (lanes 3–7) after exposure to 1 mM $HgCl_2$ for 5, 15, 30, 60 and 120 min, respectively. **a**, latent M_r 98,000 form; **a'**, first active form of **a**, M_r 88,000; **b**, latent M_r 125,000 form; **b'**, first active form of **b**, M_r 115,000; **c**, latent M_r 220,000 form; **c'**, first active form of **c**, M_r 200,000. The band pattern of lane 7 remains unchanged over a period of about 12 h, then further autolysis becomes visible by additional bands.

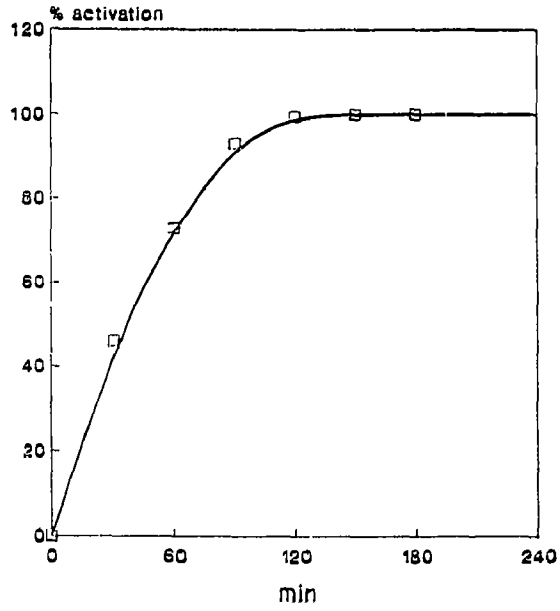

Fig. 2. Time-dependent activity of PMNL type IV collagenase during mecurial activation against DNP-octapeptide.

Time-dependent activation with $HgCl_2$ was investigated by SDS/PAGE and by determination of type IV collagenase activity as measured by proteolytic degradation of the synthetic DNP-octapeptide.

The SDS/PAGE showed that the three type IV collagenase proenzyme forms were completely transformed after 2 h into products with lower molecular masses. Determination of the activity (Fig. 2) showed that activation was complete after 2 h. The activity correlates with the formation of the products with lower molecular masses shown by SDS/PAGE. Reduction in molecular mass is therefore necessary for generation of activity. After overnight incubation with $HgCl_2$ at 37°C the activity decreased due to autolysis of the enzyme.

Since SDS/PAGE analysis of the activation was not very instructive, autoproteolytic processing of human PMNL type IV procollagenase (M_r 220,000, M_r 125,000, M_r 98,000), induced by $HgCl_2$, was analysed by RP-HPLC. This allowed separation and isolation of the reaction products which could be subjected to N-terminal sequence determination. Autoproteolytic cleavage of the enzyme and further cleavage of the liberated reaction products resulted in a time-dependent generation of various peptides.

After 5 min of $HgCl_2$-incubation four peptides could be isolated (Fig. 3, peptides I–IV). The N-terminal sequences of these peptides revealed that the three peptide bonds Asp^{15}–Leu^{16}, Glu^{40}–Met^{41} and Leu^{52}–Leu^{53} were

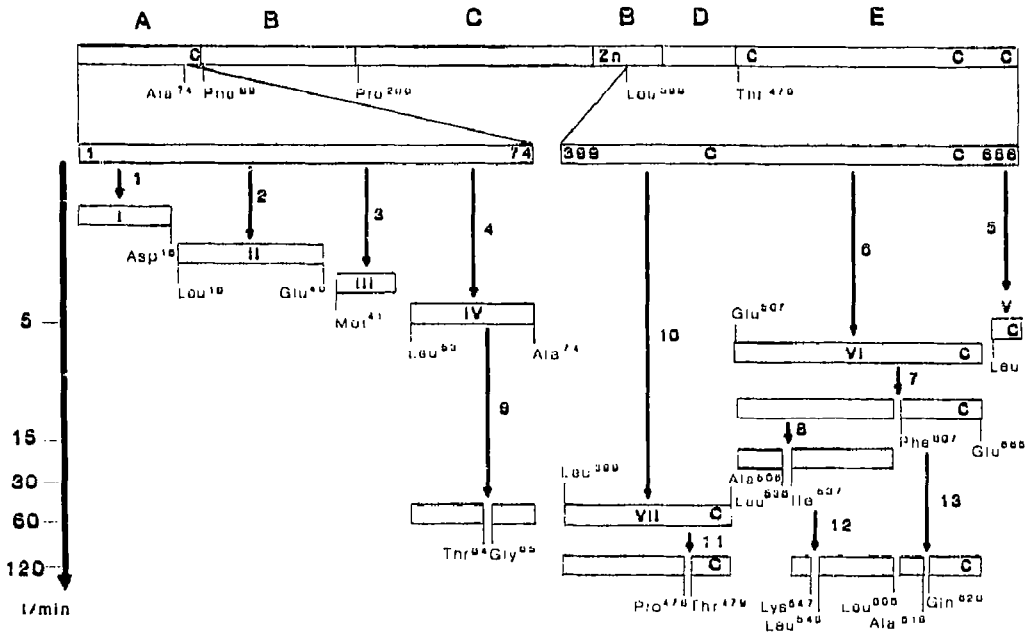

Fig. 3. Time-dependent processing of PMNL type IV procollagenase induced by $HgCl_2$. (A) propeptide; (B) catalytic domain; (C) fibronectin-like domain inserted into the catalytic domain B; (D) type V collagen-like domain; (E) hemopexin-like domain; (I–IV) propeptide fragments in the order of their appearance; (V–VII) C-terminal fragments in the order of their appearance; (1–13) chronological order of formation of the reaction products.

cleaved. Sequence determinations of the enzyme isolated by RP-HPLC after 10 min activation with $HgCl_2$ revealed the five N-terminal residues Ala^1, Leu^{16}, Met^{41}, Leu^{52} and Met^{75}. From this we concluded that the four peptides were sucessivly formed, without the loss of the PRCGVPD region, corresponding to a four-step propeptide truncation.

During the next 10 min of $HgCl_2$ incubation two further peptides were isolated (Fig. 3, peptides V and VI). Their formation resulted from the hydrolysis of the Glu^{666}–Leu^{667} and the Ala^{506}–Glu^{507} peptide bond. The Met^{75}–Glu^{666} enzyme corresponded to the M_r 88,000 and the Met^{75}–Ala^{506} enzyme to the M_r 73,000 (Fig. 1, lane 7) active type IV collagenase forms.

The next molecular mass reduction of type IV collagenase was observed in the form of the isolation of another peptide after 60 min incubation with $HgCl_2$ (Fig. 3, peptide VII). This peptide was created from cleavage of the Ala^{398}–Leu^{399} peptide bond, which resulted in the formation of the M_r 63,000 active type IV collagenase form. This could not be visualized by SDS/PAGE (Fig. 1). The isolation of these three peptides (V–VII) corresponded to a three-step C-terminal truncation of the enzyme.

For the M_r 125,000 proenzyme form the SDS/PAGE showed a loss of 10,000 Da, which corresponded to the formation of the M_r 88,000 enzyme form.

The dimer of the M_r 98,000 unit, which shows a molecular mass of 220,000 on SDS/PAGE, was successively cleaved into M_r 200,000 and 150,000 activation products. The former corresponded to the Mr 88,000 monomer and the later to the Mr 73,000 monomer.

The seven primary split products of the autoproteolytic process underwent further cleavages. During the first 15 min of $HgCl_2$ incubation four secondary split products were isolated, which resulted from the sixth primary peptide (Fig. 3, peptide VI) via hydrolysis of the Leu^{606}–Phe^{607} followed by hydrolysis of the Leu^{536}–Ile^{537} peptide bond (Fig. 3, cleavages 7 and 8). After 60 min activation with $HgCl_2$ two new propeptide fragments could be isolated. The fourth primary split product was cleaved at the Thr^{64}–Gly^{65} peptide bond (Fig. 3, cleavage 9). After the next 60 min incubation six further peptides occurred. They correspond to three cleavages of three C-terminal split products (Fig. 3, cleavages 11–13). The Pro^{476}–Thr^{479} peptide bond of the seventh primary peptide was hydrolysed. The peptide Ile^{537}–Leu^{606} was cleaved at the Lys^{547}–Leu^{548} and the peptide Phe^{607}–Glu^{666} at the Ala^{619}–Gln^{620} peptide bond.

In summary, 19 peptides were isolated during 2 h of $HgCl_2$ incubation. The main products of these peptides are the four propeptide split products (Fig. 3, I–IV) and the two C-terminal peptides (Fig. 3, V and VI), which are formed during the first 10 min.

3.2. *Disulfide bond assignment of the hemopexin-like domain*

The hemopexin-like domain of type IV collagenase contains three cysteine residues. The two cysteines,

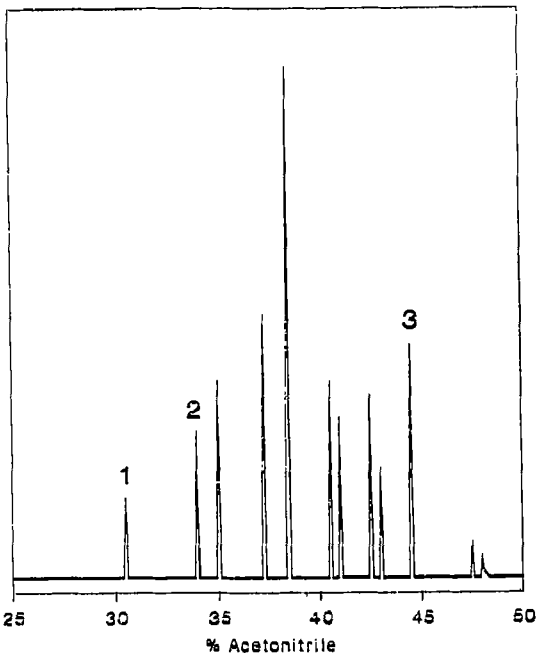

Fig. 4. Demonstration of the different retention times of the cysteine-carrying peptides RP-HPLC elution profile after 2 h exposure to 1 mM $HgCl_2$ (150 μg type IV collagenase). (1) peptide Thr^{479}-Ala^{506} contains Cys^{497}; (2) peptide Leu^{667}–Asp^{686} contains Cys^{683}; (3) peptide Glu^{507}–Glu^{666} contains Cys^{655}.

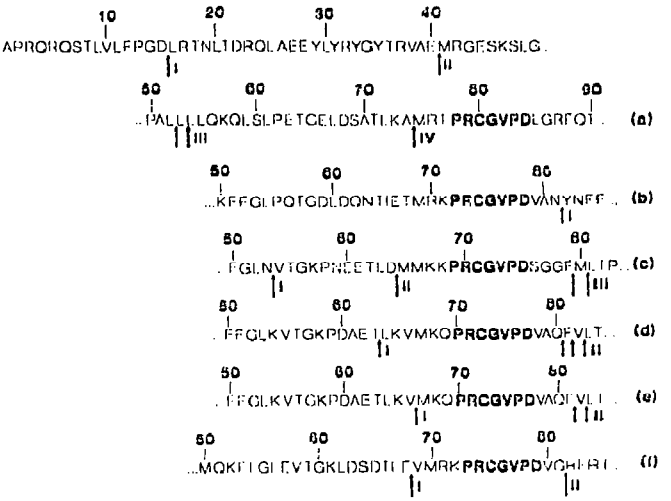

Fig. 5. Cleavages observed during activation of various metalloproteinases induced by mercurial compounds. (a) PMNL type IV collagenase; (b) M_r 72000 type IV collagenase [5]; (c) PMNL collagenase [19]; (d) fibroblast collagenase [6]; (e) fibroblast collagenase [7]; (f) stromelysin [9].

Cys^{497} and Cys^{683}, which correspond to the two conserved cysteines in the hemopexin-like domains of all metalloproteinases, are thought to be disulfide bridged [21]. Cys^{655} is an additional cysteine of the C-terminal domain of the type IV collagenase. The two peptides: Glu^{507}–Glu^{666} (contains Cys^{655}; Fig. 4, peak 2) and Leu^{667}–Asp^{686} (contains Cys^{683}; Fig. 4, peak 3) are main products of the autoproteolytic processing. The peptide Leu^{349}–Ala^{506} (contains Cys^{497}; Fig. 4, peak 1) was not isolated until after 60 min incubation with $HgCl_2$. But, all three peptides eluted under non-reducing conditions at different retention times under RP-HPLC conditions (Fig. 4, peaks 1,2,3; 30.8, 34.5, 44.5% acetonitrile)

4. DISCUSSION

Mercurial compounds possess no proteolytic activity, but they catalyse the autoproteolysis of metalloproteinases. Our results show that the activation mechanism of PMNL type IV procollagenase in vitro is very complex. A four-step truncation from the N-terminal end is followed by a three-step loss of C-terminal fragments. The PRCGVPD sequence motif remains with the rest of the enzyme. This is unique among the known activation mechanisms of different members of the matrix metalloproteinase family (Fig. 5). Fibroblast collagenase and stromelysin were autoproteolytically processed by a two-step mechanism [6,7,9]. The M_r 72,000 gelatinase followed a single-step activation mechanism but Okada et al. [5] discuss the intramolecular generation of an intermediate, so the activation is also thought to be a two-step process. Bläser et al. [19] found a three-step propeptide truncation for PMNL collagenase. These individual cleavage steps during the activation process refer only to the cleavages in the propeptide domain.

C-Terminal truncation of a metalloproteinase during activation is presumed to occur by He et al. [8] for the activation of collagenase by stromelysin. On the other hand, it could be shown that stromelysin exists in two active forms which share the same N-terminus. The M_r 45,000 form is only N-terminally truncated, whereas the M_r 28,000 active stromelysin has, in addition, lost the hemopexin-like domain [20].

Since cDNA sequences of all metalloproteinases have become available, the differences in the domain structure of these enzymes are obvious [21]. The smallest metalloproteinase, PUMP, consists of only two domains, the propeptide and the catalytic domain. In addition to this, all other metalloproteinases have a hemopexin-like, C-terminal region. Both type IV collagenases (gelatinases) possess an insertion in the catalytic domain upstream of the zinc binding motif. This fibronectin-like insertion is responsible for the gelatin binding [2]. Moreover, the M_r 98,000 type IV collagenase contains a type V collagen like sequence, which is inserted before the prolin-rich region. Another important difference between PMNL type IV collagenase and the other members of the matrix metalloproteinase family is the additional cysteine residue in the hemopexin-like domain.

We could show that under non-reducing conditions,

protein fragments are generated and separated, proving that all three cysteine residues are not disulfide bridged. Such a high amount of free cytseine residues are uncommon. If the cysteine of the PRCGVPD region of type IV collagenase is assumed to be unbridged, it might interact with other disulfide bridges of the enzyme in an intramolecular disulfide exchange reaction. This intramolecular interchange might generate the free cysteines found in the isolated peptides. However, the higher molecular mass forms of type IV collagenase, which are formed from the M_r 98,000 basic subunit of gelatinase with another M_r 98,000 unit or the unidentified M_r 28,000 protein seems to indicate one or more disulfide-bridges between these molecules. This seems to support the assumption that type IV collagenase possesses more than just one free cysteine.

Some site-directed mutagenesis experiments have confirmed the cysteine switch mechanism for transin [12,13]. The activation mechanism of type IV collagenase might be homologous to those of the other metalloproteinases. The four-step propeptide truncation without the loss of the PRCGVPD region causes a conformational rearrangement around the Zn centre resulting in an active enzyme. The C-terminal split products found correspond to the loss of the C-terminal domain of stromelysin and, therefore, have no effect on the activity of this enzyme. There are also special structural features of the type IV collagenase, a unique member of the metalloproteinases with a type V collagen homologous domain and an additional cysteine residue in the hemopexin-like domain. This, taken together with our findings of a relatively early loss of the Cys^{686} but no loss of the Cys^{80} from the PRCGVPD sequence motif, seems to indicate a mechanism of activation by intramolecular cysteine/disulfide rearrangement tha t is different from the cysteine-switch mechanism [10,11], but identical in the result by generating an accessible, catalytically active zinc at the enzyme's reactive site.

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