Direct activation of human neutrophil procollagenase by recombinant stromelysin

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Human neutrophil procollagenase was activated by incubation with recombinant active stromelysin. Activation was achieved by cleavage of the Gly⁷⁸–Phe⁷⁹ peptide bond at the end of the propeptide domain in a single-step activation mechanism. In addition, accelerated activation was achieved when N-terminally truncated, latent collagenase (with Phe⁴⁹ as its N-terminal residue) was incubated with recombinant active stromelysin. Determination of the specific activity of recombinant-stromelysin-activated neutrophil collagenase with dinitrophenyl-octapeptide or type I collagen demonstrated the generation of

high specific activity. The specific activity of stromelysin-activated enzyme was considerably higher than that of trypsin- or HgCl₂-activated collagenase. Thus human neutrophil collagenase is superactivated, like the homologous fibroblast collagenase [Murphy, Cockett, Stephens, Smith and Docherty (1987) *Biochem. J.* **248**, 265–268]. The occurrence of Phe⁷⁹ at the N-terminus of the neutrophil collagenase seemed to be critical for superactivation, which is in agreement with data published by Suzuki, Enghild, Morodomi, Salvesen and Nagase [(1990) *Biochemistry* **29**, 10261–10270] on fibroblast collagenase.

INTRODUCTION

The collagenase subfamily of matrix metalloproteinase consists of two distinct members, fibroblast or interstitial collagenase, and polymorphonuclear leucocyte or neutrophil collagenase. Their primary structures have been elucidated by analysis of their respective cDNAs, demonstrating a close structural relationship between these homologous enzymes (Goldberg et al., 1986; Hasty et al., 1990). The fibroblast proenzyme is secreted immediately after synthesis *in vitro* and *in vivo* (Nagase et al., 1983), and only a minor amount of the proenzyme is post-translationally processed by glycosylation (Wilhelm et al., 1986). In contrast, the neutrophil procollagenase is stored as a glycosylated protein within the specific granules of the neutrophils (Murphy et al., 1977; Knäuper et al., 1990a). Secretion of the neutrophil proenzyme can be initiated by inflammatory mediators *in vitro*, which trigger the cells to secrete their metalloproteinases as inactive precursors (Hasty et al., 1986). Conversion of the procollagenase into the active enzyme is the key step in the initiation of collagenolysis during the connective tissue turnover caused by inflammatory processes mediated by neutrophils. Activation can be initiated by proteinases, mercurials and oxidative processes *in vitro* (Knäuper et al., 1990a; Mookhtiar and Van Wart, 1990; Bläser et al., 1991; Michaelis et al., 1992). Activation of the proenzyme results in the removal of at least 80 or 81 N-terminal amino acid residues, thereby generating the active enzyme. To date, proteolytic as well as oxidative activation mechanisms have been assumed to be of significance *in vivo*. Cathepsin G, a neutrophil serine proteinase, activates the neutrophil procollagenase by cleavage of the Phe⁷⁹–Met⁸⁰ peptide bond at the end of the propeptide domain (Knäuper et al., 1990a). In contrast, human neutrophil elastase does not activate the enzyme, although proteolytic processing of the Asn⁵³–Val⁵⁴ peptide bond was observed, which generated a still latent N-terminally trunc-

ated enzyme (Knäuper et al., 1990a). In addition, it was demonstrated that neutrophil procollagenase is activated by oxygen radicals, which are generated during the respiratory burst of neutrophils (Weiss et al., 1985; Burkhardt et al., 1986; Saari et al., 1990).

It is generally accepted that the *in vivo* activation of fibroblast procollagenase and prostromelysin is achieved via the plasminogen/plasminogen activator/plasmin system. This cannot be the case for the neutrophil proenzymes, which are resistant to plasmin activation (Murphy et al., 1989; Knäuper et al., 1990a). The interaction between neutrophil procollagenase and recombinant stromelysin is investigated in this paper. The generation of collagenase activity is shown, and this, among others, may be one of the physiologically relevant activation pathways in the pathogenesis of rheumatoid arthritis, where both enzymes may have key roles in connective tissue turnover.

MATERIALS AND METHODS

Purification of neutrophil procollagenase and recombinant stromelysin

Neutrophil procollagenase was purified to homogeneity by established methods (Knäuper et al., 1990a,b). The purity of the enzyme preparation was demonstrated by SDS/PAGE and N-terminal sequence determination of the proenzyme, which was compared with the sequence of the cDNA (Hasty et al., 1990), revealing identity (Figure 1). Recombinant full-length stromelysin was purified as described by Housley et al. (1993).

Activation of the recombinant prostromelysin and activity assay

Activation of recombinant stromelysin was achieved by heat treatment at 55 °C for 45 min, as previously described (Koklitis et al., 1991). Low-molecular-mass recombinant stromelysin was

Figure 1 N-terminal sequence of human neutrophil procollagenase: comparison of the activation sites

The N-terminal sequence of the isolated enzyme is identical with the determined sequence of the cDNA clone according to Hasty et al. (1990). Activation sites are indicated by arrows. a, First tryptic cleavage; b, first autoproteolytic cleavage following activation by HgCl_2 ; c, second autoproteolytic cleavage following activation by HgCl_2 ; d, second tryptic cleavage; e, final active enzyme: Met⁸⁰ N-terminus following activation by trypsin; Met⁸⁰ and Leu⁸¹ N-terminus following activation by HgCl_2 (see Knäuper et al., 1990a; Bläser et al., 1991); Phe⁷⁹ N-terminus following activation by stromelysin.

thereby generated. Stromelysin-like enzymic activity was assessed by digestion of the synthetic peptide Pro-Tyr-Ala-Tyr-Trp-Met-Arg, as described (Netzel-Arnett et al., 1991). The peptide was dissolved in 20 mM Tris/HCl, pH 7.5, 5 mM CaCl_2 , 200 mM NaCl and 5% (v/v) dimethyl sulphoxide at a concentration of 400 μM . Substrate (100 μl) was incubated with 40 μl of active stromelysin (650 ng) for between 10 min and 4 h. The reaction was terminated by the addition of 100 μl of EDTA solution (100 mM, pH 7.5) and analysed by reverse-phase h.p.l.c. using a Bakerbond C_{18} column (4.9 mm $\times$ 250 mm). Integration of the signals corresponding to the cleaved substrate allowed calculation of the enzymic activity. One unit of stromelysin-like activity was defined as 1 μmol of substrate cleaved per min at 37 °C. The active recombinant stromelysin displayed a specific activity of 377 munits/mg.

Activation of neutrophil procollagenase by active recombinant stromelysin

Neutrophil procollagenase (2 μM) was activated by incubation with 1 μM or 0.2 nM active recombinant stromelysin for up to 24 h at 37 °C.

Activation of N-terminally truncated latent neutrophil collagenase by active recombinant stromelysin

Neutrophil procollagenase (0.23 μM) was treated with 4.6 nM trypsin for 5 min in order to generate the latent intermediate form (with Phe⁴⁹ at the N-terminus), as described (Knäuper et al., 1990a). The reaction was terminated by the addition of a 10-fold molar excess of bovine pancreatic trypsin inhibitor (BPTI). Neutrophil collagenase with an N-terminal Phe⁴⁹ (115 nM) was incubated in the presence of 11.5 nM active recombinant stromelysin for 5 or 15 min to obtain the final active form.

Activation of neutrophil procollagenase by HgCl_2 and trypsin

Neutrophil procollagenase (0.23 μM) was inactivated by brief treatment with 1 mM HgCl_2 for 1 h at 37 °C. Proteolytic activation of the proenzyme (0.23 μM) was achieved by treatment

with 4.6 nM trypsin (porcine) for between 15 min and 1 h at 37 °C. The reaction was terminated by the addition of a 10-fold molar excess of BPTI.

Determination of peptidolytic and collagenolytic activity

Enzymic activity was routinely determined by degradation of the synthetic octapeptide dinitrophenyl (Dnp)-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (Masui et al., 1977), whereas collagenolytic activity was determined by degradation of soluble type I collagen and analysis by SDS/PAGE followed by Coomassie Blue staining and gel scanning (Welgus et al., 1981). One unit of peptidolytic activity corresponded to the degradation of 1 μmol of substrate per min at 37 °C. One unit of collagenolytic activity was defined as the degradation of 1 μg of type I collagen per min at 25 °C.

Purification of tissue inhibitor of metalloproteinases (TIMP)-1 and recombinant TIMP-2

TIMP-1 was isolated from human rheumatoid synovial fluid (Osthues et al., 1992). Recombinant TIMP-2 was purified from the culture medium of transfected Chinese hamster ovarian cells (DeClerck et al., 1991).

SDS/PAGE analysis

SDS/PAGE was performed according to Laemmli (1970). Proteins were visualized by silver staining (Heukeshoven and Dernick, 1985).

Separation of stromelysin-activated neutrophil collagenase from recombinant stromelysin by h.p.l.c. and N-terminal sequence determination

Stromelysin-activated neutrophil collagenase was purified by reverse-phase h.p.l.c. on a Bakerbond wide-pore C_{18} column (4.9 mm $\times$ 250 mm) at a constant flow rate of 0.8 ml/min using a linear gradient from 0 to 80% acetonitrile in 60 min. The purified enzyme was freeze-dried and the N-terminal sequence was determined by automated Edman degradation using a

microsequencer (Model 810; Knauer, Berlin, Germany) (Reinke et al., 1991).

Protein determination

Protein concentrations were determined using the Bradford method (Bradford, 1976).

RESULTS

Purity and stability of neutrophil procollagenase (M_r 85 000)

To investigate the activation of neutrophil procollagenase by recombinant stromelysin, it was essential to isolate the precursor of neutrophil collagenase free of contaminating proteinases. This was achieved by a series of chromatographic steps as previously described (Knäuper et al., 1990a). The purity of the final enzyme preparation was confirmed by N-terminal sequence analysis (Figure 1); the sequence was identical to the deduced amino acid sequence of the cDNA clone characterized by Hasty et al. (1990). The enzyme was completely latent and displayed no enzymic activity against natural or synthetic substrates prior to activation. The proenzyme remained inactive when incubated in buffer for 24 h at 37 °C, thus allowing investigation of the activation mechanism during this period.

Direct activation of neutrophil procollagenase by recombinant stromelysin

Recombinant, proteolytically active, stromelysin was incubated with neutrophil procollagenase. Generation of enzymic activity was monitored versus time (Figures 2a and 2b). It could be demonstrated that neutrophil procollagenase was efficiently and directly activated by recombinant stromelysin when incubated at a molar ratio of procollagenase/recombinant stromelysin of 2:1 or 10:1. The maximal specific activity attained against Dnp-octapeptide was 2870 munits/mg after 6 h of activation. The specific activity determined was higher than that determined after activation by trypsin or HgCl_2 (Table 1). Similar results were obtained using soluble type I collagen as substrate (Table 2). The specific activity of stromelysin-activated neutrophil collagenase was 11 000 units/mg; this is considerably higher than that of the trypsin- or HgCl_2 -activated enzyme (3000 and 3300 units/mg respectively), indicating a superactivation of neutrophil procollagenase by stromelysin.

The M_r of neutrophil procollagenase (85000) was lowered upon activation by recombinant stromelysin, as demonstrated by SDS/PAGE (Figure 3). The activated enzyme showed an M_r of 64000, indicating proteolytic cleavage within the protein core of the proenzyme. Portions of partially and fully activated neutrophil collagenase were removed from the reaction mixture at the indicated time intervals (see Figures 2a and 2b) and analysed by reverse-phase h.p.l.c. prior to N-terminal sequence analysis. The kinetics of the activation reaction could be followed using this method. The time-dependent generation of a single new N-terminus could be demonstrated, indicating that recombinant active stromelysin directly cleaved the Gly⁷⁸–Phe⁷⁹ peptide bond at the end of the propeptide domain, which is essential for the generation of peptidolytic and collagenolytic activity. The generation of the Phe⁷⁹ N-terminus is the result of specific bond cleavage by recombinant stromelysin, since autoproteolysis of collagenase itself would generate Met⁸⁰ or Leu⁸¹ N-termini as previously demonstrated (Bläser et al., 1991).

The propeptide segment, which is co-ordinated via the sole cysteine within the conserved Pro-Arg-Cys-Gly-Val-Pro-Asp

sequence motif to the integral Zn centre of the proenzyme, and is essential for the latency of the matrix metalloproteinases (Holz et al., 1992), is lost upon activation of neutrophil procollagenase by stromelysin-induced cleavage of the Gly⁷⁸–Phe⁷⁹ peptide bond.

Figure 2 Activation of human neutrophil procollagenase by recombinant active stromelysin

Neutrophil procollagenase (2 μM) was incubated with 0.2 μM (▲) or 1 μM (✱) recombinant active stromelysin for the time intervals indicated. Aliquots of the reaction mixture were removed and the specific activity of the activated collagenase was determined with Dnp-octapeptide (a) or soluble type I collagen (b) as substrate.

Table 1 Specific activity of activated human neutrophil collagenase with Dnp-octapeptide as substrate

'Phe⁴⁹ collagenase' has Phe⁴⁹ as the N-terminal residue.

Collagenase	Specific activity (munits/mg)
Stromelysin-activated	2870
Trypsin-activated	1500
HgCl ₂ -activated	1800
Phe ⁴⁹ collagenase	0
Phe ⁴⁹ collagenase activated by stromelysin (5 min)	2860

Table 2 Specific activity of activated human neutrophil collagenase with soluble type I collagen as substrate

'Phe⁴⁹ collagenase' has Phe⁴⁹ as the N-terminal residue.

Collagenase	Specific activity (units/mg)
Stromelysin-activated	11 000
Trypsin-activated	3 000
HgCl ₂ -activated	3 300
Phe ⁴⁹ collagenase	0
Phe ⁴⁹ collagenase activated by stromelysin (5 min)	10 950

Figure 3 Conversion of human neutrophil procollagenase into the active enzyme by recombinant active stromelysin

Neutrophil procollagenase (2 μ M) was activated by treatment with 0.2 μ M recombinant active stromelysin at 37 °C. Aliquots were removed from the reaction mixture and the M_r s were determined by SDS/PAGE under non-reducing conditions. Lane 1, M_r markers; lanes 2–6, procollagenase in the presence of the recombinant active stromelysin for 0, 1, 2, 3 and 4 h respectively.

Thus human neutrophil procollagenase is efficiently activated by recombinant stromelysin in a single-step activation mechanism, which follows the rule of the cysteine switch activation model (Van Wart and Birkedal-Hansen, 1990; Springman et al., 1990).

Activation of N-terminally truncated latent neutrophil collagenase (Phe⁴⁹ N-terminus) by recombinant stromelysin

N-terminally truncated latent neutrophil collagenase was generated by brief trypsin treatment and was incubated in the presence of active recombinant stromelysin at a 10:1 molar ratio for 5 and 15 min. The peptidolytic and collagenolytic activities of the activated neutrophil collagenase were then determined. The specific activity of the enzyme generated by combined trypsin and stromelysin treatment was comparable with that of the superactivated enzyme following activation by stromelysin alone (results not shown). The Phe⁴⁹ N-terminus intermediate form (M_r 70 000) was converted to the final active form by extremely fast cleavage of the Gly⁷⁸–Phe⁷⁹ peptide bond, generating the M_r -

Figure 4 Accelerated activation of N-terminally truncated latent neutrophil collagenase (Phe⁴⁹ N-terminus) by co-incubation with recombinant active stromelysin

Lane 1, neutrophil procollagenase; lane 2, N-terminally truncated latent neutrophil collagenase (Phe⁴⁹ N-terminus; 115 nM) generated by trypsin treatment (5 min at 37 °C); lane 3, active neutrophil collagenase (Phe⁷⁹ N-terminus; 115 nM) generated by incubation with trypsin in the presence of recombinant active stromelysin (11.5 nM) (5 min at 37 °C); lane 4, neutrophil procollagenase (115 nM) in the presence of recombinant active stromelysin (11.5 nM) (5 min at 37 °C). The non-dissociated trypsin/BPTI complex migrates at M_r 35 000, indicated by an arrow.

Figure 5 Inhibition of the recombinant active stromelysin (0.2 μ M)-induced activation of human neutrophil procollagenase (2 μ M) by either TIMP-1 or recombinant TIMP-2

Lane 1, M_r markers; lane 2, activated neutrophil collagenase after 4 h in the presence of recombinant active stromelysin; lane 3, inhibition of the activation by preincubation of recombinant active stromelysin with TIMP-1; lane 4, inhibition of the activation by preincubation of recombinant active stromelysin with recombinant TIMP-2; lane 5, neutrophil procollagenase in the presence of buffer (latent control). The migration positions of TIMP-1 (top) and recombinant TIMP-2 (bottom) are indicated by arrows. The protein band of M_r 30 000 in lanes 2, 3 and 4 corresponds to the catalytic domain of stromelysin.

64 000 collagenase (Figure 4). Cleavage of the Gly⁷⁸–Phe⁷⁹ peptide bond at the end of the propeptide domain seems to be the rate-limiting step during activation of neutrophil collagenase by recombinant stromelysin.

It can be assumed that, if intermediates are generated during stromelysin activation of human neutrophil procollagenase, immediate proteolytic conversion to the final active form by removal of the remaining propeptide segment would follow. The cleavage of the Gly⁷⁸–Phe⁷⁹ peptide bond itself does not involve collagenase, and is definitely due to stromelysin-specific cleavage.

Inhibition of neutrophil procollagenase activation by preincubation of active recombinant stromelysin with TIMP-1 and TIMP-2

Preincubation of active recombinant stromelysin with TIMP-1 or recombinant TIMP-2 suppressed the decrease in M_r , thus indicating stromelysin-specific cleavage (Figure 5). Furthermore, both TIMP-1 and recombinant TIMP-2 suppressed this activation process, indicating both TIMP-1 and TIMP-2 can regulate the activity of neutrophil collagenase at the activation level and at the level of the active enzyme.

DISCUSSION

Activation of neutrophil procollagenase is one of the critical control mechanisms of connective tissue turnover mediated by neutrophils. We have demonstrated that recombinant stromelysin directly activates neutrophil procollagenase in a single-step activation mechanism. This might be of physiological relevance in the pathogenesis of rheumatoid arthritis, where elevated levels of stromelysin and neutrophil collagenase are observed (Bergmann et al. 1989; Obata et al., 1992; McDonnell et al., 1992). In addition, stromelysin bound to collagen fibrils (Allan et al., 1991) could serve as an effective activator of neutrophil procollagenase within the connective tissue itself, thus leading to locally activated collagenase.

The propeptide of neutrophil collagenase is removed by stromelysin-specific cleavage of the Gly⁷⁸-Phe⁷⁹ peptide bond, accompanied by the expression of collagenolytic activity. Activation is rapid, and is achieved by incubation of neutrophil procollagenase with active recombinant stromelysin at a 2:1 or 10:1 molar ratio. In contrast, direct activation of the homologous fibroblast procollagenase could only be attained by incubation with a 200-fold molar excess of stromelysin within 20 h (Suzuki et al., 1990). The peptide bond cleaved (Gln⁸⁰-Phe⁸¹) within the fibroblast procollagenase sequence was obviously only poorly accessible to stromelysin. The reaction was extremely slow, even when a 20-fold molar excess of stromelysin was used. However, treatment of either partially activated N-terminally truncated fibroblast collagenase (M_r 43000) or latent N-terminally truncated neutrophil collagenase (M_r 70000) with stromelysin at a 1:10 or 10:1 molar ratio resulted in accelerated activation of fibroblast collagenase or neutrophil collagenase. The Gln⁸⁰-Phe⁸¹ peptide bond of fibroblast collagenase and the Gly⁷⁸-Phe⁷⁹ peptide bond of neutrophil collagenase were more susceptible to stromelysin, leading to accelerated activation and generating enzymes displaying high specific activity.

In the case of the neutrophil enzyme, the Gly⁷⁸-Phe⁷⁹ peptide bond seems to be more readily accessible to stromelysin, allowing rapid proenzyme activation in a single step, although activation via very short-lived intermediate forms cannot be excluded. The differences in the accessibility of the peptide bond cleaved might possibly be due to the amino acid sequence of the propeptide domain or to slightly different proenzyme folding, which could be caused by glycosylation of the propeptide of the neutrophil enzyme.

Activated neutrophil collagenase displayed high specific activity, comparable with the data published on fibroblast collagenase by Murphy et al (1987) and Suzuki et al (1990). They also observed superactivation of the enzyme in the presence of active stromelysin. The reason why high specific activities are obtained by activation by stromelysin either alone or in the presence of plasmin, mercurials or other activators is unknown to date, but it was assumed that the generation of the Phe⁸¹ N-terminus is critical for the generation of high specific activity of fibroblast collagenase. In addition, it has been suggested that proteolytic cleavage within the C-terminal domain of fibroblast

collagenase molecule takes place (He et al., 1989), resulting in a fully active enzyme conformation; however, there is no experimental evidence to support this. Our sequence data failed to demonstrate any such C-terminal processing. The generation of the Phe⁷⁹ N-terminus seemed to be critical for superactivation of neutrophil collagenase as well, and even after a prolonged incubation time (24 h), high specific collagenolytic activity was observed. The activated neutrophil collagenase (Phe⁷⁹ N-terminus) was more stable than the trypsin- or HgCl₂-activated enzyme (Met⁸⁰ or Leu⁸¹ N-termini), since fragmentation, which results in the loss of collagenolytic activity (Knäuper et al., 1993), was suppressed.

The activation of human neutrophil procollagenase by stromelysin is the second example of matrix metalloproteinase activation directly triggered by another member of the matrix metalloproteinase family. Recently, Ogata et al. (1992) showed that stromelysin is an effective activator of high- M_r procollagenase (M_r 92000) from HT1080 cells, which is immunologically and structurally identical to the neutrophil enzyme (Devarajan et al., 1992). A two-step activation mechanism was observed, which was triggered by catalytic amounts of stromelysin. Thus activation of human neutrophil matrix metalloproteinases by stromelysin seems to be a generally observed phenomenon.

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