RESEARCH COMMUNICATION

The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases

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Recombinant 72 kDa gelatinase A and a truncated form lacking the C-terminal domain were shown to be activated by organomercurials and to possess similar activities towards a number of substrates. The truncated proenzyme differed from the full-length gelatinase in that it could not be activated by a membrane activator and did not bind tissue inhibitor of metalloproteinase (TIMP)-2. Kinetic studies also showed that the inhibition of the activated truncated enzyme, by both TIMP-1 and TIMP-2, was considerably decreased compared with the full-length enzyme. We conclude that the C-terminal domain plays an important role in the regulation of gelatinase A by a potential physiological activator and inhibitors.

INTRODUCTION

Comparisons of the primary structures of the matrix metalloproteinases (MMP) have shown that they can be divided into distinct domains (Docherty & Murphy, 1990; Murphy *et al.*, 1991a). The propeptide and catalytic domain are common to all known enzymes of the family and, with the exception of the punctuated metalloproteinase, PUMP (MMP7), a C-terminal domain with sequence similarities to hemopexin and vitronectin can be defined. The role of the latter domain is of interest, since it has been shown to be essential for interstitial collagenase (MMP1) substrate specificity (Birkedal-Hansen, *et al.*, 1988; Clark & Cawston, 1989), but is not required for stromelysin-1 (MMP3) activity (Okada *et al.*, 1986; Koklitis *et al.*, 1991). It apparently mediates collagen binding of both these enzymes (Allan *et al.*, 1991; Murphy *et al.*, 1992). Gelatinase A and B (MMP2 and MMP9) consist of the three common domains described above, as well as a domain with similarity to the gelatin-binding region of fibronectin; the 95 kDa gelatinase B (MMP9) has a further collagen-like insertion (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). Studies on the 72 kDa gelatinase A (MMP2; EC 3.4.24.24) have shown that it has several unique properties of potential importance for its biological regulation. The secreted proform is not activated by proteinases such as plasmin or kallikrein (Okada *et al.*, 1990; Hipps *et al.*, 1991), but a membrane activator can be induced on the surface of both normal and transformed cells (Brown *et al.*, 1990; Ward *et al.*, 1991a). The proform of 72 kDa gelatinase A binds tissue inhibitor of metalloproteinase (TIMP)-2 to form a complex of 1:1 stoichiometry (Stetler-Stevenson *et al.*, 1989), whereas all other MMPs can only bind this inhibitor in the active form. Furthermore, active gelatinase A can in some instances show impaired inhibition by TIMP-1 (Howard *et al.*, 1991a). To investigate the

role of the gelatinase A C-terminal domain, we have prepared a deletion mutant from which this domain has been completely omitted [$\Delta_{418-631}$ gelatinase (N-GL); i.e. the enzyme coded for by gelatinase A cDNA lacking nucleotides 418–631]. Comparison of the properties of this mutant with the wild-type gelatinase A has provided a basis for the understanding of some of the unique properties of this enzyme.

METHODS

Preparation of recombinant human 72 kDa progelatinase A and N-GL

Cloning of human gelatinase A. A human gelatinase A cDNA was isolated from a mixed human tumour cDNA library in λ gt10 (kindly given by Dr. R. Breathnach, Université de Nantes, Nantes, France; Muller *et al.*, 1988). The *EcoRI* cDNA fragment was subcloned into the vector pSP65, and its nucleotide sequence was determined by the dideoxy-chain-termination method (Sanger *et al.*, 1977). This cDNA was found to be identical with that described by Collier *et al.* (1988) for this enzyme, but was truncated at the 5' end, starting at nucleotide 76 and terminating with a poly(A) tail. The 5' end of the gene was rebuilt using a series of overlapping complimentary oligonucleotides with a sequence based on the 5' coding sequence of the gelatinase gene (Huhtala *et al.*, 1990; A. J. P. Docherty, C. Catterall, P. Slocombe & C. Gafton, unpublished work). After DNA sequencing (Sanger *et al.*, 1977) to confirm that the resulting DNA encoded gelatinase A, as reported by Collier *et al.* (1988) and Huhtala *et al.* (1990), the DNA was ligated into plasmid pEE12 (Murphy *et al.*, 1991c) to create the gelatinase A expression vector pEE12-GL A.

Construction of N-GL. The conversion of adenosine-418 into a stop codon followed by a *BamHI* site was achieved by using pEE12-GL A as template DNA and PCR (Mullis & Faloona,

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; N-GL, $\Delta_{418-631}$ gelatinase; Mca-PLGLDnpAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH₂; APMA, 4-amino-phenylmercuric acetate; EHS, Engelbreth-Holt-Swarm.

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1987; Ho *et al.*, 1989). The DNA was subjected to 20 rounds of amplification with *Taq* polymerase in the presence of an oligonucleotide of the sequence 5'-GACGACCGCAAGTGGGCT-TCT-3' that primes from within the gelatinase A coding sequence and a second mutagenic oligonucleotide encoding a stop codon followed by a *Bam*HI site: 5'-GCGCGCGGATCCGGTC-AATGTCAGGAGACTACCCATAGAGCTCCTGAATG - 3'. In order to construct an N-GL expression vector, the amplified product was digested with *Bgl*II and *Bam*HI and the resultant fragment was ligated to a *Hind*III to *Bgl*II 1270 bp fragment of the gelatinase A cDNA and a 5' *Hind*III-*Bcl*II-digested pEE12 to form the vector pEE12-delta₄₁₈₋₆₃₁ GL. The sequence of the PCR-generated fragment was shown by the dideoxy-chain-termination method to correspond to that of wild-type gelatinase, except for the C-terminal deletion achieved by insertion of the stop codon described above.

Expression of progelatinases in NSO cells. The vectors pEE12-GL A and pEE12 delta₄₁₈₋₆₃₁ GL were transfected into NSO mouse myeloma cells by electroporation as previously described (Murphy *et al.*, 1991c). Colonies isolated 2–3 weeks after transfection were screened for secretion of gelatinase A or N-GL by analysis of conditioned medium generated from selected colonies on substrate gels and in activity assays. The most productive cell lines were used to produce conditioned medium from which the recombinant wild-type and N-GL were purified.

The recombinant enzymes were purified from the NSO-cell-conditioned culture media by chromatography on gelatin-Sepharose and elution with 10% dimethyl sulphoxide (Ward *et al.*, 1991b). Small fragments were removed from the eluate by Amicon Diaflo concentration using a PM10 membrane and chromatography on Sephacryl S-200. The major peak of protein eluted corresponded to the gelatinolytic activity, and these fractions were pooled.

Purification of natural human progelatinase A

Natural human progelatinase A was purified from human-gingival-fibroblast-conditioned medium essentially as described by Ward *et al.* (1991b), except that TIMP-2–progelatinase complexes were separated from free progelatinase by using heparin-Sepharose (Kolkenbrock *et al.*, 1991).

Proteinase activity

Gelatinases were activated by incubation with 2 mM-4-aminophenylmercuric acetate (APMA) at 25 °C for 1.5 h (see the Results section).

Activity against gelatin was determined by using ¹⁴C-labelled type I collagen denatured at 60 °C for 20 min (Murphy *et al.*, 1981a). One unit is defined as 1 µg of gelatin degraded/min at 37 °C. Degradation of soluble Engelbreth–Holt–Swarm (EHS) type IV collagen, kindly given by Professor K. Kühn, Max Planck Institut für Biochemie, Munich, Germany, was qualitatively assessed by PAGE (Murphy *et al.*, 1991c). Kinetic studies were carried out using (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-ArgNH₂ (McaPLGLDnpAR; kindly given by Dr. G. Knight, Strangeways Research Laboratory; Knight *et al.*, 1992) in the presence of 50 mM-Tris/HCl (pH 7.4)/100 mM-NaCl/10 mM-CaCl₂/0.05% Brij 35. Enzyme concentration was determined by titration with TIMP-2 after preincubation of enzyme (approx. 50 nM) with TIMP-2 (2–70 nM) for 4 h at 25 °C. It was established that TIMP-2 inhibited the active forms of both enzymes at 1:1 molar stoichiometry (Ward *et al.*, 1991a; F. Willenbrock, unpublished work). Assays were then performed using an enzyme concentration of 0.5–1 nM.

TIMP inhibition of gelatinase activity was assessed by preincubation of active enzymes and inhibitors (0–50 nM) at 25 °C

for 30 min or 12 h before the addition of gelatin substrate. Estimates of *K_i* values were obtained using 2.5 µM-Mca-PLGLDnpAR, 20 pM active gelatinase A or N-GL and 2–200 pM-TIMPs. Enzyme and inhibitor were preincubated at 50–100 times the above concentrations at 25 °C overnight. They were then diluted to a total volume of 2.5 ml in assay buffer and incubated for a further 8 h before assay. Data was analysed using the equation:

$$V_s = \frac{V_0}{2[E_i]} \{[(K_{i(\text{app.})} + [I_i])^2 + 2(K_{i(\text{app.})} - [I_i])[E_i] + [E_i]^2]^{1/2} - (K_{i(\text{app.})} + [I_i] - [E_i])\} \quad (\text{Morrison \& Walsh, 1988})$$

In the case of the TIMP-binding study, TIMP activity was also assessed in the standard rabbit collagenase assay (Murphy *et al.*, 1981b).

Activation of progelatinases by fibroblast membrane preparations

Crude fibroblast plasma-membrane preparations were prepared from concanavalin A-stimulated human fibroblasts as described by Ward *et al.* (1991a). The progelatinase-activating potential of these preparations was determined by incubating aliquots of natural or recombinant progelatinase A (138 ng) or pro-N-GL (45 ng) with various amounts of membrane preparation for 16 h at 37 °C in a final volume of 40 µl. Maximal gelatinase activity was taken as that obtained by activating with 2 mM-APMA for 2 h at 25 °C. Activation with 10 µM-APMA for 16 h at 37 °C was carried out for comparative purposes.

TIMP-binding studies

TIMP-2 was incubated with an approx. 0.5 M excess of progelatinases (30–50 pmol) in a volume of 50 µl [25 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl/10 mM-CaCl₂/0.05% Brij 35] for 1 h at 25 °C. Incubation mixtures were loaded on to 100 µl gelatin-Sepharose columns in pipette tips and washed with 3 × 50 µl of the same buffer. The total wash of 200 µl was pooled. Columns were washed with a further 500 µl of the same buffer and this was discarded. The columns were eluted with 3 × 50 µl of the same buffer containing 15% dimethyl sulphoxide and the eluates were pooled.

Gel-electrophoretic analyses

Purified gelatinases were analysed by SDS/PAGE under reducing conditions (Laemmli & Favre, 1973), followed by silver (Merril *et al.*, 1981) or Coomassie Blue staining. The same protocol was followed for the analysis of type IV collagen degradation.

Gelatin zymogram analysis of both gelatinases and TIMPs in the TIMP-binding and activation studies were carried out as described by Murphy *et al.* (1991b), with 0.3 mg of gelatin/ml in a 12% polyacrylamide gel. Electrophoresis was in the presence of SDS and in the absence of reducing agents.

RESULTS AND DISCUSSION

Recombinant progelatinases A and its N-terminal truncated form N-GL were purified from NSO-myeloma-cell culture media using gelatin-Sepharose followed by Sephacryl S-200 chromatography. The enzymes were homogeneous, as assessed by reducing SDS/PAGE, with molecular masses of 72 kDa and 46 kDa respectively (Fig. 1). Both gelatinases were totally inactive in the proform and quite stable, but autoactivated in the presence of the organomercurial APMA, with a similar time course to yield species of 66 kDa and 36 kDa respectively (Fig. 1). We have previously shown that natural progelatinase A exists in a stable inactive proform in the absence of TIMP-2 (Ward *et al.*, 1991b). This is in contrast with the findings of Howard *et al.* (1991a) in

Fig. 1. Activation of progelatinase A and pro-N-GL

Progelatinase A and pro-N-GL were incubated in the presence of 2 mM-4-APMA at 25 °C and sampled at time intervals with addition of 20 mM-EDTA before electrophoresis on an SDS/polyacrylamide gel under reducing conditions. Pro-N-GL is shown in lane 1, after 30 min incubation (lane 2), 60 min incubation (lane 3) and 120 min incubation (lane 4). Progelatinase A is shown in lane 5, after 30 min incubation (lane 6), 60 min incubation (lane 7) and 120 min incubation (lane 8). Over this time period the proenzymes incubated in the absence of organomercurial did not undergo any decrease in molecular mass. The molecular masses (*M*) of standard proteins are indicated to the right of the gel.

which they demonstrate that acid-treated progelatinase, freed from its complex with TIMP-2, is unstable and autoactivates. Studies in our laboratories suggest that gelatinase is very sensitive to low pH, which causes denaturation and auto-degradation. The specific activity for the degradation of ^{14}C -labelled gelatin was 464 units/nmol for active gelatinase A and 583 units/nmol for active N-GL, comparable with that of natural full-length gelatinase (Ward *et al.*, 1991b; Hipps *et al.*, 1991). Both forms of gelatinase displayed similar activity against solubilized EHS-sarcoma type IV collagen, generating the typical 'specific clip' pattern of approx. 145, 130 and 90 kDa products (Fig. 2; Fessler *et al.*, 1984; Murphy *et al.*, 1989). When assayed with the peptide substrate McaPLGLDnpAR, similar $k_{\text{cat.}}/K_m$ values were observed: $5.7 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for active gelatinase A and $5.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for active N-GL. Hence the gelatinase A is comparable with stromelysin-1, which does not appear to require its C-terminal domain to degrade either macromolecular or peptide substrates (Okada *et al.*, 1986; Koklitis *et al.*, 1991; Marcy *et al.*, 1991; G. Murphy, unpublished work). However, this domain could be required for other, as yet undefined, substrates.

We have previously shown that plasma-membrane preparations from concanavalin A-stimulated human fibroblasts were able to activate both endogenous natural progelatinase A and exogenous recombinant progelatinase A (Ward *et al.*, 1991a). In the present study we confirmed that membrane preparations were able to activate both recombinant and natural progelatinase A to about 80 % of the maximal activity of the APMA-treated enzyme. In similar experiments, fibroblast membrane preparations were unable to activate the pro-N-GL to greater than 2 % of the activity of the APMA-treated enzyme (Fig. 3). Electrophoretic analysis demonstrated that, where plasma-membrane

Fig. 2. Type IV collagen degradation by active gelatinase A and N-GL

Soluble type IV collagen derived from the EHS sarcoma (20 μg) was incubated at 28 °C alone (lane 1) or in the presence of active gelatinase A (2.9 pmol, lane 2) or active N-GL (2.9 pmol lane 3). The incubation mixtures were analysed by SDS/PAGE and silver staining. The molecular masses (*M*) of standard proteins and of the α - and β -chains of type I collagen are indicated.

Fig. 3. Analysis of the effect of fibroblast membrane preparations on progelatinase A and pro-N-GL

Samples of progelatinases (138 ng of progelatinase A or 45 ng of pro-N-GL) were incubated with or without aliquots of fibroblast membrane preparation or APMA before analysis on a 7.5%-polyacrylamide gel containing SDS and gelatin. Assays for gelatinase activity were also performed. Gelatinase activities are expressed at the top of each lane as a percentage of the activity obtained by activating equivalent aliquots of progelatinase with APMA. The samples were: lane 1, progelatinase A incubated with 10 μM -APMA for 16 h at 37 °C; lane 2, pro-N-GL incubated with 2 mM-APMA for 1.5 h at 25 °C. Aliquots of pro-N-GL were incubated with the following amounts of fibroblast membrane preparation for 16 h at 37 °C: lane 3, 36.8 μg ; lane 4, 24.6 μg ; lane 5, 12.3 μg ; lane 6, no membrane preparation. Aliquots of progelatinase A were also incubated for 16 h at 37 °C with the same amounts of membrane preparation: lane 7, 36.8 μg ; lane 8, 24.6 μg ; lane 9, 12.3 μg ; lane 10, no membrane preparation. Endogenous gelatinase A can be observed by incubating aliquots of membrane preparation alone: lane 11, 36.8 μg ; lane 12, 24.6 μg ; lane 13, 12.3 μg . Differences in the sizes of the zones of clearing between the forms of gelatinase are due to variations in their migration patterns. The molecular masses (*M*) of standard proteins are indicated.

preparations could almost fully convert the progelatinase A into 59 kDa and 43 kDa active forms in 16 h (non-reducing conditions), the pro-N-GL remained unchanged as the proenzyme

Fig. 4. Binding of TIMP-2 to progelatinase A and pro-N-GL

TIMP-2 (1 μ g) was incubated with an 0.5 molar excess of progelatinase A or pro-N-GL at room temperature for 1 h in a volume of 50 μ l. Each component was also incubated alone. The incubation mixtures were loaded on to 100 μ l gelatin-Sepharose columns and washed, the pooled washes representing unbound material as described in the Methods section. The columns were eluted with 15% (v/v) dimethyl sulphoxide to give the bound fraction. Both groups of fractions were assayed for TIMP activity, and the amount in each pool (assessed as a percentage of activity in the original TIMP-2 added to the incubation mixture), is given at the top of each lane. Portions of each fraction were electrophoresed on an SDS/polyacrylamide gel containing gelatin, and the enzyme and inhibitor content revealed as described in the Methods section. Lanes 1 and 6, TIMP-2 alone unbound (1) and bound (6); lanes 2 and 7, pro-N-GL unbound (2) and bound (7); lanes 3 and 8, TIMP-2 and pro-N-GL unbound (3) and bound (8); lanes 4 and 9, TIMP-2 and progelatinase A unbound (4) and bound (9); and lanes 5 and 10, progelatinase A alone unbound (5) and bound (10). Both forms of gelatinase show bands of the activated material by this method, which is not quantitative, and no active enzyme could be detected by enzymic assay.

with the appearance of minor lower-molecular-mass forms (Fig. 3).

The ability of pro-N-GL to bind TIMP-2 relative to progelatinase A was compared. TIMP-2 was incubated with either of the two enzyme forms, or alone, before chromatography on gelatin-Sepharose. The elution of free TIMP-2 activity was assayed, demonstrating that TIMP-2 bound to progelatinase A and was retained on gelatin-Sepharose, whereas TIMP-2 in the presence of pro-N-GL, or alone, did not bind. The gelatinases were eluted from gelatin-Sepharose and analysed by reverse zymography (Fig. 4), which confirmed that TIMP-2 had bound to the matrix only in the presence of full-length proenzyme. These data show that TIMP-2 interacts with 72 kDa progelatinase through the C-terminal domain of the enzyme. Howard & Banda (1991) also showed that a 29 kDa C-terminal fragment of gelatinase A binds TIMP-2. Interestingly, we have also found that TIMP-1 and TIMP-2 interact, through their C-terminal domains, with 95 kDa and 72 kDa progelatinases B and A respectively (Murphy *et al.*, 1991b; G. Murphy, unpublished work).

The fact that the C-terminal domain is required for membrane activation suggests that, during activation, the proenzyme may interact through this domain with some component of the cell membrane. Since TIMP-2 binds to this domain in the proenzyme

Fig. 5. Inhibition of gelatinases by TIMP-1 and TIMP-2

Active gelatinase A (a) and active N-GL (b) were preincubated with increasing amounts of TIMP-1 (●) and TIMP-2 (○) for 30 min of TIMP-1 (■) and TIMP-2 (□) for 12 h at room temperature before 14 C-labelled-gelatin assay at 37 °C for 1 h. The percentage inhibition of the enzyme relative to its activity after preincubation in the absence of TIMP is shown as the mean ($\pm$ S.E.M.) of four replicates.

and has also been shown to be an efficient inhibitor of membrane activation (Ward *et al.*, 1991a), we propose that a role for TIMP-2 *in vivo* may be the blocking of enzyme-membrane interactions, thereby preventing cell-associated gelatinase A activation.

The ability of TIMPs to interact with active forms of the gelatinases was assessed. The inhibitions of fully activated gelatinase A and N-GL by TIMP-1 and TIMP-2 were compared by using 14 C-labelled gelatin (Fig. 5). Whereas the full-length enzyme was equally well inhibited by TIMP-1 and TIMP-2, the N-GL form was more weakly inhibited by TIMP-2 and could not be inhibited by TIMP-1 after short preincubations of 30 min (Fig. 5). The equivalent activity of TIMP-1 and TIMP-2 against active natural gelatinase A was demonstrated previously (Ward *et al.*, 1991b) and contrasts with the results of Howard *et al.* (1991a), who found TIMP-2 a more efficient inhibitor. The modification of TIMP inhibition of the enzyme after removal of the C-terminal domain indicates the involvement of this domain in TIMP-active-enzyme interaction. Similar data were obtained by Howard *et al.* (1991b), who showed that active fragments of gelatinase A lacking the C-terminal domain, generated autocatalytically, were less well inhibited by TIMP-1 than TIMP-2. However, in our studies, after preincubation of the inhibitors with N-GL overnight, TIMP-1 and TIMP-2 were of similar efficacy (Fig. 5).

With a synthetic substrate, McaPLGLDnpAR, active gelatinase A was inhibited with a K_i of about 2 pM for both TIMPs. Owing to limitations in the sensitivity of the assay, it was not possible to work at the low enzyme concentrations required to obtain

accurate K_i values. Under the conditions used, however, similar inhibition curves were observed, suggesting that these values are comparable. Analysis of the inhibition of active N-GL gave K_i values of 63 μM for TIMP-1 and 33 μM for TIMP-2. The rates of inhibition of 20 μM enzymes by 20 nM-TIMPs were compared by using the synthetic substrate to monitor the reaction. Whereas both TIMPs fully inhibited active gelatinase A within 30 s, complete inhibition of active N-GL by TIMP-2 and TIMP-1 was only observed after approx. 40 min and 10 h respectively. These data are consistent with the results obtained using the gelatin assay.

The similar affinities of both TIMPs for gelatinase suggests that the TIMP-binding sites on the active enzyme are common to both inhibitors. The fact that only TIMP-2 binds to progelatinase indicates that binding to the proenzyme occurs by a different mechanism and that there are therefore two distinct TIMP-binding sites on the C-terminal domain; one is unique to TIMP-2 and functional in the proenzyme and the other interacts with both TIMPs in the active enzyme. An important contribution of the C-domain to the inhibition of active gelatinase by TIMP-1 and TIMP-2 is to increase the rate of binding. It can be envisaged that an initial rapid, but relatively low-affinity, interaction between the C-terminal domain of gelatinase with either TIMP results in a conformational change in the catalytic domain which increases the accessibility of the active site to the inhibitory portion of the TIMP molecule.

The fact that the TIMPs have a decreased rate of reaction with, and affinity for, N-GL, may be of great significance in biological systems where autocatalytically generated low-molecular-mass active forms of gelatinase A (Howard *et al.*, 1991b), if structurally similar to the N-GL used here, may escape the regulatory surveillance of the tissue inhibitors.

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