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Large inhibitor of metalloproteinases (LIMP) contains tissue inhibitor of metalloproteinases (TIMP)-2 bound to 72000-M_r progelatinase

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Connective-tissue cells in culture produce a family of metalloproteinases which, once activated, can degrade all the components of the extracellular matrix. These potent enzymes are all inhibited by the tissue inhibitor of metalloproteinases (TIMP), and it was thought that this inhibitor was solely responsible for the inhibition of these enzymes within connective tissue. However, other inhibitors have recently been described, including large inhibitor of metalloproteinases (LIMP) present in the culture medium of human foetal lung fibroblasts. Here we show that a large proportion of the inhibitory activity of LIMP consists of 72000-M_r-progelatinase bound to TIMP-2, a recently discovered low-M_r metalloproteinase inhibitor closely related to TIMP. The physiological implications of the secretion of a complex of 72000-M_r progelatinase and TIMP-2 are discussed, and the separation of the complex in 6 M-urea is described.

INTRODUCTION

The matrix metalloproteinases (MMPs) are an important family of enzymes, produced by connective-tissue cells, which between them can degrade all the components of the extracellular matrix [1]. A number of different control mechanisms exist to control the extracellular activity of these potent enzymes. These include the control of the synthesis and secretion of the enzymes, the existence of proenzyme forms that require activation before substrate can be degraded, and the presence of metalloproteinase inhibitors.

The naturally occurring metalloproteinase inhibitors have important physiological roles in a wide variety of situations where connective-tissue turnover is taking place [2,3]. The plasma protein α_2-macroglobulin inhibits all the four classes of proteinases and it accounts for > 95% of the anti-metalloproteinase activity of human plasma [4]. A tissue-derived glycoprotein is found in body fluids and culture media conditioned by cells and tissues and has been identified in a variety of different tissues and species. This inhibitor (M_r 28000) is specific for vertebrate metalloproteinases and was named 'Tissue Inhibitor of Metallo-Proteinases' (TIMP) [5]. This protein has been sequenced and expressed in recombinant form [6,7] and shown to contain six disulphide bonds [8].

High-M_r metalloproteinase inhibitors have been described in rheumatoid synovial fluid [9], bovine endothelial-cell culture medium [10] and rabbit chondrocyte culture medium [11].

De Clerck *et al.* [12] subsequently purified the high-M_r inhibitor in the culture medium of bovine endothelial cells and showed that, although it shared many properties with TIMP, it was not glycosylated and was of M_r 20400. This decrease in size was interesting, as the initial gel-filtration column had separated a high-M_r peak of inhibitory activity that had been used as the starting material for the purification. Stetler-Stevenson *et al.* [13] described a similar inhibitor secreted by human melanoma cells which they named 'TIMP-2'. This protein has been cloned and sequenced. The amino acid sequence of TIMP-2 revealed significant sequence identity with TIMP, with the conservation of all 12 cysteine residues. Interestingly TIMP-2 is often found in culture medium in a non-covalent complex with 72000-M_r progelatinase [13–15].

In a recent study [16] we showed that a 76000-M_r inhibitor is present in human lung fibroblast culture medium. This large inhibitor of metalloproteinases (LIMP) inhibited the three metalloproteinases collagenase, gelatinase and stromelysin, forming tight-binding complexes. It was not a multimeric form of TIMP. In the present study we have further characterized LIMP and show that a large proportion of the inhibitory activity consists of TIMP-2 in combination with 72000-M_r progelatinase.

MATERIALS AND METHODS

Chemicals were obtained from the following suppliers: gelatin–Sepharose from Pharmacia, Milton Keynes, U.K.; *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (1M 861; Bolton and Hunter reagent) from The Radiochemical Centre, Amersham, Bucks, U.K.; Coomassie Brilliant Blue G250 from BDH, Poole, Dorset, U.K.; antibody to TIMP-2 was kindly given by Professor Yves DeClerck, Division of Hematology/Oncology, Childrens Hospital of Los Angeles, Los Angeles, CA 90027, U.S.A. All other chemicals and biochemicals were commercially available analytical-grade reagents or have been previously described [5,17,18].

Enzyme assays

[^{3}H]Acetylated collagen was used to measure collagenolytic activity by the diffuse-fibril assay [19]. Gelatin-degrading activity was assayed using ^{3}H-labelled gelatin at 37 °C as previously described [1].

Protein determination and radioiodination

Column eluates were monitored for protein by measuring A_{280}. Protein samples were iodinated using Bolton and Hunter reagent as previously described [16].

SDS/PAGE

SDS/PAGE was performed as previously described [16]. The polyacrylamide gels were photographed, dried and radioauto-

Abbreviations used: TIMP, Tissue Inhibitor of MetalloProteinases; LIMP, Large Inhibitor of MetalloProteinases; MMP, matrix metalloproteinases.
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graphed [17]. Western blotting and gelatin substrate gels were as described by Clark & Cawston [20]. For the detection of inhibitory activity against gelatinase, proteins were separated on gelatin-containing gels as described above; the gels were incubated with active gelatinase (10 units/ml) for 1 h at room temperature, then in 100 mM-Tris/HCl/10 mM-$CaCl_2$/0.02% NaN_3, pH 7.9, for 16 h at 37 °C [21]. After staining, inhibitory bands were revealed as a zone of stained undegraded gelatin against a clear background.

Column chromatography

Gelatin–Sepharose columns were equilibrated with 25 mM-sodium cacodylate buffer, pH 7.5, containing 0.5 M-NaCl, 10 mM-$CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3. Samples were applied in this buffer, and the bound proteins were eluted with the same buffer containing 10% (v/v) dimethyl sulphoxide. Gel-filtration columns (Superdex 75; 1.6 cm × 60 cm) were equilibrated with the above buffer and run by using an f.p.l.c. system (Pharmacia–LKB, Milton Keynes, U.K.).

Separation of progelatinase and TIMP-2

Samples of progelatinase–TIMP-2, ^{125}I-labelled with Bolton and Hunter reagent [16], were dialysed into a series of buffers containing chaotropic reagents or of high or low pH and were then separated on f.p.l.c. Superdex columns equilibrated with the same buffer at a flow rate of 0.5 ml/min. Fractions were collected, and the amount of ^{125}I-labelled protein in each fraction was determined by using an LKB γ-radiation counter. The buffers used in this study were: 50 mM-glycine/HCl, pH 3.0, containing 10 mM-$CaCl_2$, 0.02% NaN_3 and 0.05% Brij (Buffer A); 25 mM-borate, pH 10.0, containing 10 mM-$CaCl_2$, 0.02% NaN_3 and 0.05% Brij (Buffer B); 20 mM-Tris/HCl, pH 7.5, containing 50 mM-NaCl, 0.02% NaN_3 and 0.1% SDS (Buffer C); Buffer A + 10 mM-EDTA (Buffer D); 25 mM-sodium cacodylate, pH 7.6, containing 1 M-NaCl, 0.05% Brij, 0.02% NaN_3, 10 mM-$CaCl_2$ and 6 M-urea (Buffer E); 25 mM-sodium cacodylate, pH 7.6, containing 1 M NaCl, 0.05% Brij, 0.02% NaN_3, 10 mM-$CaCl_2$ and 20 mM-EDTA (Buffer F).

Immunoblotting using antibody to TIMP-2

Proteins were separated by SDS/PAGE and electroblotted on to nitrocellulose paper. This was then incubated with rabbit anti-(bovine TIMP-2) IgG, followed by horseradish-peroxidase-conjugated pig anti-(rabbit IgG) antibody (Dako). Colour was developed with 4-chloro-1-naphthol.

RESULTS

The fractions containing LIMP activity from a Superdex 75 f.p.l.c. gel-filtration column were pooled and applied to a gelatin–Sepharose column. Some inhibitory activity against collagenase was found in the fractions that passed through the column; protein eluted from the column with 10% dimethyl sulphoxide also contained inhibitory activity (Fig. 1).

This gelatin–Sepharose binding pool was concentrated in a CentriPrep 10 concentrator (Amicon) and radioiodinated with Bolton and Hunter reagent. After reduction the protein was separated by SDS/PAGE. The gel was dried and autoradiographed. Two proteins were present, one of M_r 72000 and one of M_r 21000, in approximately equal amounts (Fig. 2). In all purified preparations the ratio of 72000-M_r protein to 21000-M_r protein is approx. 1:1 when viewed by autoradiography. The 21000-M_r protein does not appear to stain well with Coomassie Brilliant Blue. However, we have noted that the ratio of gelatinolytic activity to inhibitory activity varies markedly between different preparations.

The binding pool was applied to a gelatin-substrate gel. A clear lysis zone was observed at M_r 72000 and 66000, corresponding to the M_r of pro- and active 72000-M_r gelatinase respectively (Fig. 3*a*). This activity was inhibited by *o*-phenanthroline, indicating that it was a metalloproteinase. Incubation of the gelatin-containing gel overnight with partially purified gelatinase removed most of the gelatin from the gel, apart from around the 21000-M_r protein band (lane 1), showing that this protein inhibited gelatinase (Fig. 3*b*). Purified human TIMP was loaded in lane 2 as a control, and the inhibition of lysis zone around this protein is indicated by the arrow (Fig. 3*b*, lane 2).

Identification of TIMP-2 by immunoblotting

A sample of the gelatin–Sepharose binding pool was separated by SDS/PAGE and electroblotted on to nitrocellulose paper. After incubation with rabbit anti-(bovine TIMP-2) IgG followed by horseradish-peroxidase-conjugated pig anti-(rabbit IgG) anti-

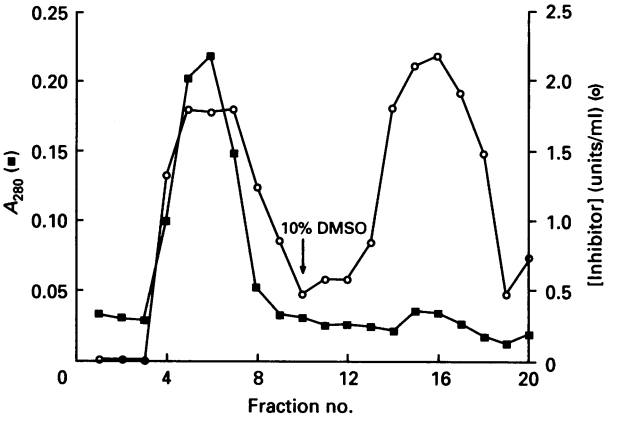

Fig. 1. Gelatin–Sepharose chromatography of LIMP fractions after gel filtration

Gel-filtration fractions containing LIMP were pooled and dialysed against 25 mM-sodium cacodylate buffer, pH 7.6, containing 10 mM-$CaCl_2$, 0.5 M-NaCl, 0.05% Brij 35 and 0.02% NaN_3 and loaded on to a column of gelatin–Sepharose (2.5 cm × 1 cm) at a flow rate of 20 ml/h. Fractions (1 ml) were collected, and the bound protein eluted by the inclusion of 10% (v/v) dimethyl sulphoxide (DMSO) in the above buffer. Fractions were monitored for protein (A_{280}, ■) and collagenase inhibitor (○).

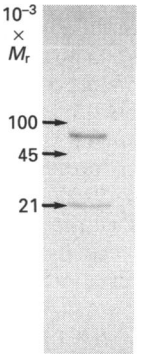

Fig. 2. SDS/12.5%-PAGE of ^{125}I-labelled gelatin–Sepharose-binding fractions followed by autoradiography

A sample of the gelatin-Sepharose binding peak was radioiodinated and reduced with dithiothreitol (4 mg/ml); gels were fixed, dried and autoradiographed. The positions of M_r markers (phosphorylase, M_r 100000; ovalbumin, 45000; soya-bean trypsin inhibitor, 21000) are shown.

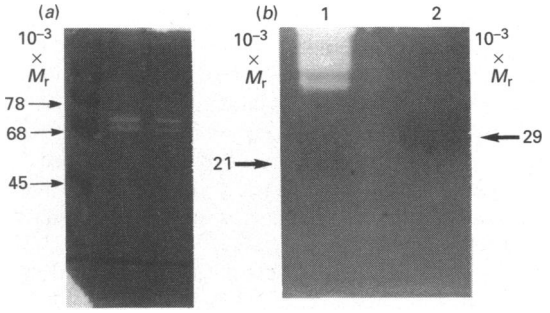

Fig. 3. Electrophoresis on a substrate gel incorporating 1 mg of type 1 gelatin/ml

(*a*) A sample eluted from the gelatin-Sepharose column was separated on a gelatin-containing acrylamide gel. Clear lysis zones indicate gelatinolytic activity, which is present in the 72000-M_r and 66000-M_r bands. M_r markers are also shown. (*b*) A sample of the gelatin–Sepharose-binding peak was separated on a gelatin-containing acrylamide gel. After electrophoresis and equilibration in Triton X-100-containing buffer to renature the proteins, the gel was digested with active gelatinase (10 units/ml) at 37 °C overnight and stained. The stained zone of undigested gelatin around the 21000-M_r band (lane 1) demonstrated that this protein inhibited gelatinase. A TIMP control is shown in lane 2. M_r markers were: transferrin, 78000; BSA, 68000; ovalbumin, 45000.

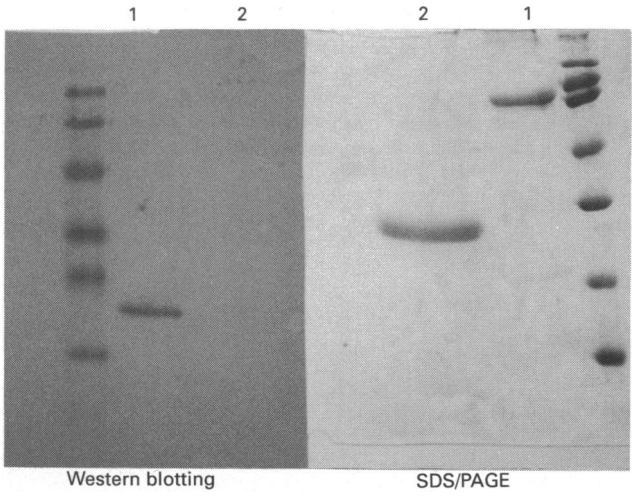

Fig. 4. Identification of TIMP-2 after separation from progelatinase by SDS/PAGE and Western blotting

A sample of the gelatin–Sepharose-binding peak was separated on either an SDS/polyacrylamide-gel and fixed and stained, or on an SDS/polyacrylamide gel followed by electroblotting on to nitrocellulose paper. This was incubated with rabbit anti-(bovine TIMP-2) IgG, followed by horseradish-peroxidase-conjugated pig anti-(rabbit IgG) antibody and the colour developed with 4-chloro-1-naphthol. Lane 1 contains progelatinase–TIMP-2; lane 2 contains the TIMP standard. The 21000-M_r band was clearly recognized, indicating that it was identical with TIMP-2. Antibody to TIMP-2 does not cross-react with TIMP.

body the blot was developed with 4-chloro-1-naphthol. The 21000-M_r protein was clearly recognized by the anti-(bovine TIMP-2) antibody (Fig. 4), thus identifying this protein as TIMP-2.

Separation of progelatinase–TIMP-2 by gel filtration

Samples of ^{125}I-labelled progelatinase–TIMP-2 were dialysed against a series of buffers to determine whether the complex could be split and the components recovered after gel filtration.

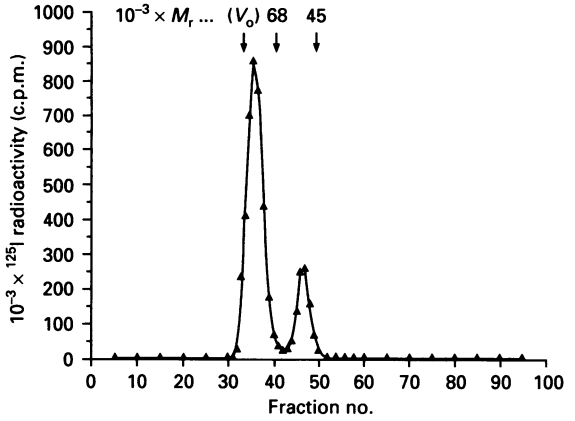

Fig. 5. Separation of ^{125}I-labelled progelatinase–TIMP-2 complex by gel filtration on f.p.l.c. Superdex 75

A sample of ^{125}I-labelled progelatinase–TIMP-2 complex, dialysed against Buffer E, was applied to a Superdex 75 (16/60) column equilibrated the same buffer at a flow rate of 0.5 ml/min. Fractions (1.35 ml) were collected and the amount of ^{125}I present in each was measured with a γ-radiation counter.

The buffers used, Buffers A–F, contained a variety of chaotropic reagents and detergents. Two peaks of radioactivity were eluted from gel filtration when the sample was pretreated with 6 M-urea (Buffer E), one with an apparent M_r of 80000 (peak 1) and another with an apparent M_r of 50000 (peak 2) (Fig. 4). After concentration over a PM10 membrane, SDS/PAGE of these peaks and subsequent autoradiography confirmed that peak 1 contained a mixture of 72000-M_r and 21000-M_r bands and peak 2 contained solely a 21000-M_r band. This indicated that the treatment with 6 M-urea had removed some, but not all, of the 21000-M_r TIMP-2 from the progelatinase (results not shown). Similar results were obtained with 0.1% SDS-containing columns (Buffer F), except that three peaks of activity were eluted. The peaks were very broad and overlapped, but SDS/PAGE showed that the highest-M_r peak (M_r 80000) contained just the progelatinase and the two lower-M_r peaks, essentially TIMP-2. The results suggested that, in the presence of SDS, a dimer of TIMP-2 could form as well as the monomer. All of the other conditions tested, which included pH 3.0 (Buffer A), pH 10.0 (Buffer B), heating at 60 °C in the presence of 20 mM-EDTA (Buffer F), pH 3.0 + EDTA (Buffer D), failed to remove TIMP-2 from the progelatinase, and a single peak of M_r approx. 80000 was eluted from the Superdex f.p.l.c. column.

DISCUSSION

A number of different groups have described high-M_r inhibitors in concentrated connective-tissue culture medium from chondrocytes [11], bovine endothelial cells [12], rheumatoid synovial fluid [9] and foetal-lung fibroblast [16]. Recently De Clerck and colleagues [12] purified a protein from bovine endothelial cells with M_r 20400. This protein and its human counterpart have been cloned and sequenced [22], and the latter is identical with that described by Stetler-Stevenson and colleagues in size and amino acid sequence [13,14]. Stetler-Stevenson *et al.* [13] purified the human melanoma-cell inhibitor, named TIMP-2, by gelatin–Sepharose chromatography; this inhibitor was tightly bound to 72000-M_r progelatinase. Goldberg *et al.* [15] also reported that 72000-M_r progelatinase purified from human epithelial cells and lung and skin fibroblasts was complexed with TIMP-2 in the culture medium. A number of recent studies have purified this protein from mouse tumour cells [23],

human hepatoma cells [24], human rheumatoid synovial cells [25] and human transformed fibroblasts [26,27].

In the present study we investigated the inhibitor we named 'LIMP' to see if it was similarly a complex of TIMP-2 bound to progelatinase. We found that LIMP contains a 21000-M_r inhibitor bound to progelatinase and that this inhibitor appears identical with TIMP-2. The immunoblotting experiments confirmed this conclusion. It is interesting to note from our previous studies that this complex is inhibitory to other active metalloproteinases and will bind to the active form of collagenase to form a tertiary complex consisting of progelatinase, TIMP-2 and collagenase [16]. A recent study by Kolkenbrock *et al.* [25] has shown that human rheumatoid synovial cells produce TIMP-2 complexed with progelatinase and that this complex acts as an inhibitor to collagenase (MMP1) and neutrophil collagenase (MMP8). Those authors also showed that progelatinase–TIMP-2 could form a tertiary complex, stable to gel filtration with the neutrophil gelatinase, and suggested that it was possible that LIMP was a complex of progelatinase and TIMP-2.

We were surprised at the results of experiments aimed at separating progelatinase and TIMP-2. Neither high or low pH appeared to separate the two components, even in the presence of EDTA, a metal chelator that should disrupt the structure of progelatinase. Heating at 60 °C in the presence of EDTA was equally ineffective. Partial separation was achieved with 6 M-urea and complete separation with SDS. These results indicate that the binding of progelatinase to TIMP-2 is extremely tight and, even if the structure of progelatinase is disrupted by heating in the presence of EDTA, still no separation is obtained.

As TIMP-2 bound to progelatinase is still inhibitory, it suggests that the binding site directed towards the active site of the metalloproteinases is different from that responsible for binding to the proenzyme. It is obvious from the recent studies by Howard *et al.* [26,27] that TIMP-2 does not bind to the propeptide, as it is still attached to the gelatinase after activation and loss of the propeptide. Further cleavage of the gelatinase can occur to give lower-M_r active forms of the enzyme that still bind TIMP-2, and in our own studies we have seen such complexes in the fractions eluted from the gel-filtration columns (results not shown). The MMP-active-site-binding region of TIMP has recently been localized to the *N*-terminal domain [28] with the binding region to 95000-M_r progelatinase located in the *C*-terminal domains. It is possible that TIMP-2 has similar properties, with the 72000-M_r progelatinase-binding portion being located in the *C*-terminal domain.

Some inhibitory activity in the LIMP preparation passed straight through the gelatin–Sepharose column. This inhibitor was not TIMP, since it came from a high-M_r pool on gel filtration. No inhibitory activity bound to the gelatin–Sepharose column on re-application. This could represent TIMP-2 dissociated from progelatinase, although our own studies, along with those from other workers, have reported that the complex is very difficult to dissociate. Alternatively a third metalloproteinase inhibitor could be present, and this requires investigation. It may be that there are at least three metalloproteinase inhibitors which, although capable of inhibiting all the members of the MMP family, will preferentially bind to and inhibit one.

The physiological relevance of a metalloproteinase inhibitor being secreted tightly bound to the proenzyme form of an enzyme that it can inhibit is intriguing. The metalloproteinases are not necessarily co-ordinately expressed, although in many situations collagenase and stromelysin are produced together, whereas gelatinase is under separate control. In some circumstances the growth factors that stimulate the production of metalloproteinase inhibitors also stimulate gelatinase production [29]. Connective-tissue breakdown may involve two well-defined stages: (1) limited removal of the matrix from around the cell, allowing it to move or repair; this is likely to take place close to the cell membrane and be tightly controlled by the level of TIMP; (2) more extensive breakdown, particularly if basement membrane has to be breached; this would be harder to control and so, in addition to secreting gelatinase as a proenzyme, a complex with a specific inhibitor is released. This would restrict the activity of the active enzymes and so prevent uncontrolled connective-tissue breakdown. TIMP-2 may also prevent activation of 72000-M_r progelatinase [30], providing a further level of control.

The tight binding of the TIMP-2 to progelatinase is also difficult to understand from a physiological point of view. As active enzymes appear to form a tertiary complex with the progelatinase–TIMP-2, it does not seem that the TIMP-2 is released from the progelatinase to act as a free inhibitor. It is possible that, when enzyme interacts with substrate, then the interaction with TIMP-2 is weakened and the TIMP-2 can be released. Interestingly some workers [26] report that the interaction between TIMP-2 and progelatinase is much tighter if purified as a complex when compared with complexes reconstituted from purified enzyme and inhibitor.

Although progelatinase and TIMP-2 are often produced as a complex, there is no doubt that some cells can produce progelatinase free of TIMP-2. The recent description of TIMP-2-free progelatinase produced by rheumatoid synovial cells may indicate that absence of TIMP-2 may be related to an increase in the breakdown of connective tissues.

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