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Human gelatinase/type IV procollagenase is a regular plasma component

Tapio Vartio and Marc Baumann+

Departments of Pathology and +Medical Chemistry, University of Helsinki, Helsinki, Finland

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Gelatin zymograms revealed in human plasma a constant 66 kDa proteolytically active polypeptide. In most plasma samples other major proteolytic activities were seen at M_r 92000, 130000 and 225000. All four proteases were Ca^{2+}-dependent metalloproteases and bound quantitatively to gelatin-Sepharose. Immunoblotting results indicated that the 66 kDa protease was the human fibroblast gelatinase/type IV procollagenase and that the other three proteases were macrophage/granulocyte-derived gelatinase components. The 66 kDa protease did not bind to conA- nor lentil lectin-Sepharose allowing its separation from the 92, 130 and 225 kDa proteases. During the isolation procedure the plasma gelatinase/type IV procollagenase tended to form a proteolytically active spontaneous disulfide-bonded dimer and a 62 kDa component that could also be obtained by digestion with trypsin. The same polypeptide changes occurred also in stored preparations of the corresponding protease isolated from fibroblast culture medium while the freshly purified protein contained only the 66 kDa proform.

Gelatinase; Procollagenase, type IV; Plasma protein

1. INTRODUCTION

Degradation of connective tissue and basement membranes takes place in inflammation, tumor invasion and metastasis. Several types of enzymes that degrade different extracellular matrix components have been implicated in these events. Among them are the metalloproteases, such as collagenase (matrix metalloendoprotease-1; MMP-1; nomenclature in [1]), gelatinase (MMP-2) and stromelysin (MMP-3). Especially degradation of type IV collagen has been in the focus of interest and activity of enzymes capable of degrading this major basement membrane component have been correlated with metastatic potential of tumor cells in various in vitro tests (for references see [2]). One of the best characterized extracellular matrix-degrading enzymes is a gelatinase/type IV procollagenase [3] that apart from many malignant cultured cell types is also produced by a variety of normal cells [3,4]. Under nonreducing conditions in SDS-PAGE this enzyme has a proenzyme form of 66 kDa that can be activated to a 62 kDa component capable of degrading type IV collagen [3,5]. A metalloprotease that may be involved in tissue destruction in inflammatory conditions is the macrophage/granulocyte gelatinase [6–12]. The macrophage gelatinase is composed of two polypeptides of 225 and 92 kDa [13] whereas the granulocyte one contains an additional immunologically cross-reactive 130 kDa component [12,13].

The actual significance of these metalloproteases is not known and so far they have not been identified in any defined in vivo situation. We now show that the 66 kDa gelatinase/type IV procollagenase is a fairly regular constituent of normal human plasma suggesting an in vivo role for the enzyme.

Correspondence address: T. Vartio, Department of Pathology, Haartmaninkatu 3, SF-00290 Helsinki, Finland

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2. MATERIALS AND METHODS

2.1. *Plasma and proteins*

Whole blood was obtained from healthy donors and clotting was prevented by 0.38% sodium citrate. Plasma was separated from blood cells by centrifugation and stored at −20°C until analysed. Gelatin-binding proteins were isolated from plasma by gelatin-Sepharose 4B (Pharmacia, Uppsala, Sweden; [14]). The bound material was eluted with electrophoresis sample buffer with or without 2-mercaptoethanol. In some experiments the gelatin-Sepharose bound proteins were eluted with 8 M urea and dialysed in PBS (0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.4) supplemented with Ca^{2+} and Mg^{2+} and then applied to conA- or lentil lectin-Sepharose (Pharmacia) columns equilibrated with the same buffer. Lectin-Sepharose-bound material was eluted with 400 mM methyl-α-D-mannopyranoside (Calbiochem, La Jolla, CA). In a set of experiments 2 μl of aliquots of plasma (in 40 μl of PBS) were treated with gelatin-, conA- or lentil lectin-Sepharoses (vol. 20 μl). After 1 h incubation at room temperature with gentle mixing the gelatin-Sepharoses were washed three times with 1 ml vols of 50 mM Tris-HCl, pH 7.5, or with various concentrations of NaCl or urea (in the same buffer) as indicated using a 1-h incubation in each washing. The lectin-Sepharoses were similarly incubated and washed with PBS. The material which was still bound to Sepharoses after washing was then eluted with electrophoresis sample buffer. The fibroblast gelatinase/type IV procollagenase was isolated by gelatin-Sepharose from serum-free fibroblast culture medium as previously described [3,4]. Before gelatin-Sepharose experiments, the starting materials were pretreated with unconjugated Sepharose 4B to remove nonspecifically binding proteins. Digestion with trypsin (180 U/mg; Worthington, England) was performed at 37°C for 1 h with 1 U/ml of the enzyme.

2.2. *SDS-polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS-PAGE [15] was performed using acrylamide concentration of 3.3% in the spacer gel and 6% in the separating gel. The samples were run nonreduced or reduced with 10% 2-mercaptoethanol. Zymography [12,13] was performed in SDS-PAGE using gels containing 2 mg/ml gelatin. After electrophoresis the gels were incubated either in the presence or absence of 5 mM Ca^{2+}. In the latter case diethylenetriaminepentaacetic acid (EDTA, 5 mM) or 1,10-phenanthroline (20 μg/ml) were included in the solutions. In a set of experiments, *N*-ethylmaleimide (5 mM) or phenylmethylsulfonyl fluoride (1 mM) were included in zymography gel incubation solutions. All these reagents were from Sigma, St. Louis, MO.

2.3. *Immunoblotting*

In immunoblotting [16] polypeptides separated by SDS-PAGE were transferred electrophoretically to nitrocellulose sheets (type I HAWP filter, Millipore, Bedford, MA). The polyclonal rabbit antisera against human fibroblast gelatinase/type IV procollagenase and macrophage/granulocyte gelatinase were as described [4,6,13]. Immunoreactions were detected by using peroxidase-coupled swine anti-rabbit IgG antiserum (Dakopatts, Glostrup, Denmark).

3. RESULTS AND DISCUSSION

Gelatin zymograms of nonreduced proteins from 2 μl aliquots of different human plasma samples revealed a constant major proteolytically active 66 kDa polypeptide (fig.1A). Additional major active polypeptides were detected at M_r 92 000, 130 000 and 225 000. All four proteins bound quantitatively to gelatin-Sepharose (fig.1B). No proteolytic activity was seen when plain Sepharose 4B-bound plasma proteins were analysed in gelatin zymograms or when gelatin-bound proteins were analysed reduced with 2-mercaptoethanol (not shown). The proteolytic activity was dependent on the Ca^{2+} present in the zymography gel incubation solutions and no activity was seen if EDTA was substituted for the ion (not shown). Inhibition was also obtained with 1,10-phenanthroline but not with *N*-ethylmaleimide nor phenylmethylsulfonyl fluoride. These experiments indicate that all proteolytically active gelatin-binding plasma proteins are metalloproteases.

Fig.2A shows immunoblotting of the gelatin-binding proteins from 100 μl of the plasma sample of lane 4, fig.1A (denoted as plasma-4 in the following text), by anti-fibroblast gelatinase/type IV procollagenase serum. Immunoperoxidase reaction of the nonreduced sample (lane 1) was at M_r 66 000 and of the reduced sample (lane 2) at M_r 70 000. Immunoblotting of similar samples with the antiserum against the macrophage/granulocyte gelatinase showed immunoperoxidase reactions at M_r 92 000, 130 000 and 225 000 (fig.2B, lane 1, nonreduced) and M_r 95 000 (fig.2B, lane 2, reduced) corresponding to the previously published polypeptides of this metalloprotease [12,13].

Elution of the gelatin-Sepharose-bound plasma metalloproteases with NaCl or urea did not result in satisfactory separation of any of them from each other. All four proteins remained bound to the Sepharose after washing with NaCl up to 3 M (fig.3A, lane 2). Washing of Sepharose particles with 2 M urea eluted most of the proteases except the 225 kDa one (lane 3) while 4–8 M urea eluted all the gelatin-binding proteins (lanes 4–6). However, it was found that neither conA- nor lentil lectin-Sepharose bound the 66 kDa protease while others were retained by the Sepharoses (fig.3B, lanes 1 and 2). The 66 kDa protease was then separated from the other plasma metalloproteases

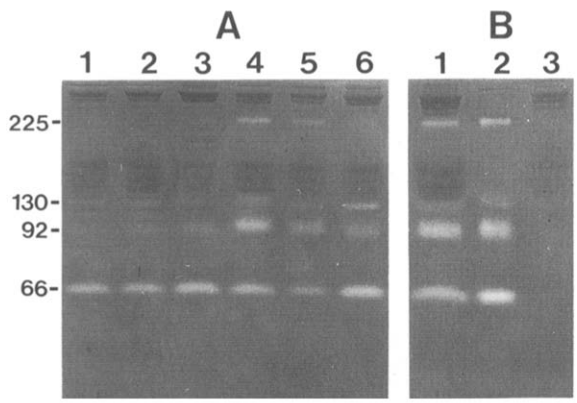

Fig.1. Gelatin zymograms of plasma proteins. (A) Zymogram of 2 μl aliquots of six different human plasma samples (lanes 1–6) run nonreduced in a 6% gelatin-containing SDS-PAGE. (B) Zymogram of the same plasma sample as in A, lane 4 (plasma-4, lane 1); gelatin-binding fraction of the same material (lane 2); the nongelatin-binding fraction of the same material (lane 3). The numbers on the left indicate molecular masses in kDa.

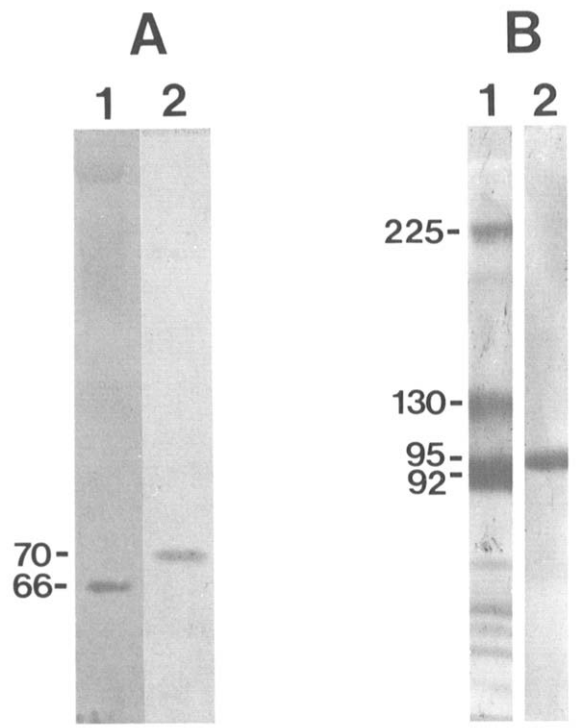

Fig.2. Immunoblotting of plasma metalloproteases. The gelatin-binding proteins isolated from 100 μl of plasma-4 were electrophoretically transferred from a 6% SDS-PAGE to nitrocellulose sheets that were immunoblotted by anti-human fibroblast gelatinase/type IV procollagenase (A) or anti-human macrophage/granulocyte gelatinase (B). Lanes 1, nonreduced and lanes 2, reduced materials. The numbers on the left of each panel indicate molecular masses in kDa.

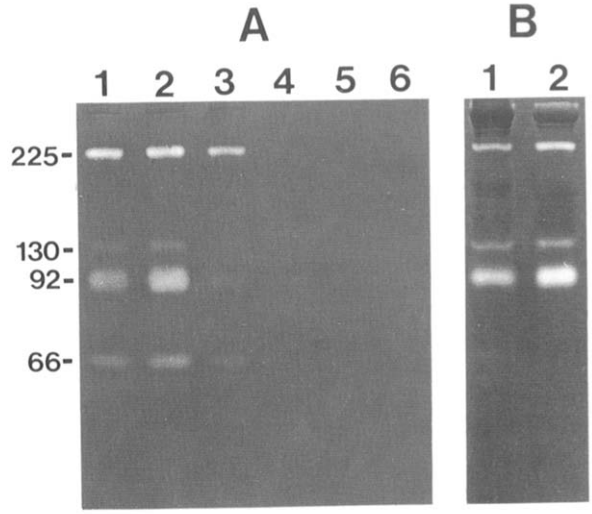

Fig.3. Zymograms of gelatin- and lectin-Sepharose-bound plasma proteins. (A) Proteins from 2 μl of plasma-4 were treated with gelatin-Sepharose (vol. 20 μl) and the elution of the bound proteins were tested by washing the Sepharose pellets with 50 mM Tris-HCl, pH 7.5 (lane 1), 3 M NaCl (lane 2), 2 M urea (lane 3), 4 M urea (lane 4), 6 M urea (lane 5) or 8 M urea (lane 6) using 1 h incubation with each washing. The material remaining bound to gelatin-Sepharose after the washings was then eluted by nonreducing electrophoresis sample buffer and analysed in a 6% gelatin-zymography gel. (B) Proteins from 2 μl of plasma-4 were treated similarly with conA-Sepharose (lane 1) or lentil lectin-Sepharose (lane 2). The Sepharoses were washed with PBS and the bound materials were eluted and analysed in a gelatin zymography gel as above. The numbers on the left indicate molecular masses in kDa.

by passing the proteins eluted from gelatin-Sepharose through a lentil lectin-Sepharose column. Zymogram in fig.4 shows the 92, 130 and 225 kDa proteases eluted from the lentil lectin-Sepharose with methyl-α-D-mannopyranoside (lane 1) and the flow-through containing frequently three active polypeptide bands at 62, 66 and 132 kDa (lane 2). Sometimes the 66 kDa protease was obtained intact in the lentil lectin-Sepharose flow-through as shown in lane 3. Treatment of this material with trypsin resulted in the formation of the 62 kDa active polypeptide (lane 4).

The three polypeptides or occasionally two of them were frequently seen also in fibroblast gelatinase/type IV procollagenase preparations that were stored for up to several months and had been freezed and thawed several times (fig.5, lanes 1 and 2). In immunoblotting all these three active polypeptides reacted with anti-fibroblast

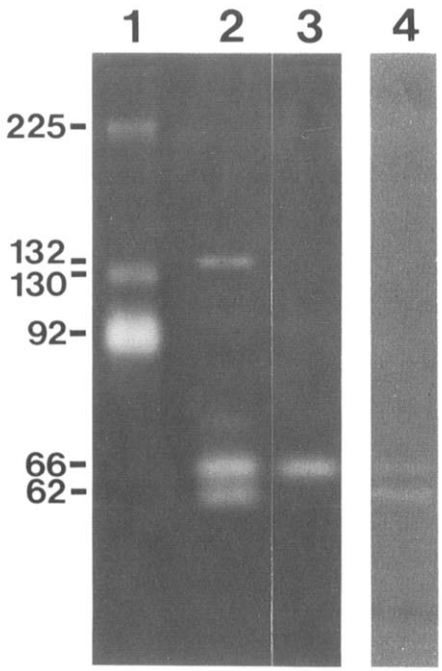

Fig.4. Zymograms of isolated plasma metalloproteases. Gelatin-binding proteins isolated from plasma-4 were passed through a lentil lectin-Sepharose column. Lanes: 1, the material eluted with methyl-α-D-mannopyranoside from the lentil lectin-Sepharose; 2, the lentil lectin-nonbound material; 3, another preparation of lentil lectin-nonbound material; 4, the material of lane 3 treated with trypsin. The numbers on the left indicate molecular masses in kDa.

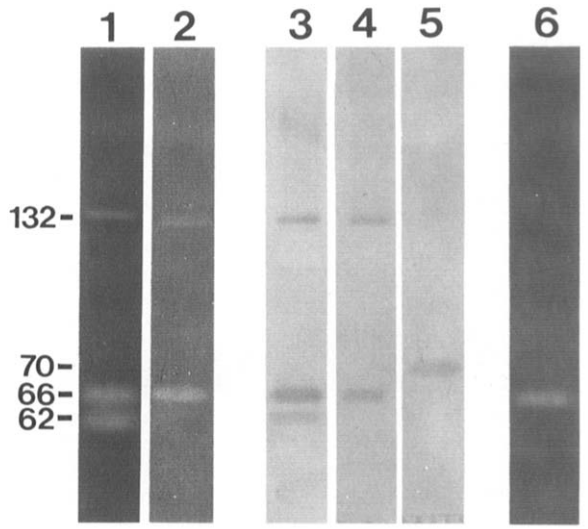

Fig.5. Zymograms and immunoblottings of fibroblast gelatinase/type IV collagenase. Zymograms of two preparations of human fibroblast gelatinase/type IV procollagenase isolated from serum-free fibroblast culture medium by gelatin-Sepharose and stored for several months (lanes 1 and 2), immunoblotting of the nonreduced material shown in lane 1 (lane 3) and in lane 2 (lane 4) with anti-fibroblast gelatinase/type IV procollagenase serum. Lane 5 shows the material of lane 2 immunoblotted reduced. Lane 6, preparation of human fibroblast gelatinase/type IV procollagenase isolated freshly from serum-free fibroblast culture medium by gelatin-Sepharose. The numbers on the left indicate molecular masses in kDa.

gelatinase/type IV procollagenase serum (fig.5, lanes 3 and 4). Immunoperoxidase reaction of the reduced material of lane 2 showed only one polypeptide band at 70 kDa (fig.5, lane 5) suggesting that the 132 kDa polypeptide was a dimer of the 66 kDa protease. Fig.5, lane 6 shows that fibroblast gelatinase/type IV procollagenase isolated freshly by using gelatin-Sepharose only, contained a single 66 kDa polypeptide. Thus, the 66 kDa gelatinase/type IV procollagenase spontaneously forms a disulfide-bonded dimer during storage or more complicated isolation procedure. Furthermore, under the same conditions the protease may undergo partial cleavage to the 62 kDa form that corresponds to the activated type IV collagenase [5] that can be obtained by treatment with trypsin as reported earlier [17].

The origin of the 66 kDa protease in plasma is difficult to ascertain since it is secreted by various normal and malignant cultured cells [3,4]. Several lines of evidence speak for its involvement in the degradation of basement membranes during tumor invasion (for references see [2]). The protease has been shown to be secreted by H-ras oncogene-transformed human bronchial epithelial cells but not by their normal counterparts [3]. Furthermore, the secretion of the protease in human fibroblasts is increased by transforming growth factor-β [18]. According to the amino acid sequence data the protease appears to be identical to the type IV procollagenase from human melanoma cells ([3,5], our unpublished results).

While the expression of the 66 kDa gelatinase/type IV procollagenase seems to be connected with malignant transformation and tumor invasion, its significance in plasma of healthy donors is unknown. It would, however, be important to find out whether the quantity of the protease in plasma would change as a reflection of any

physiologic or pathologic situation. Obviously the same concerns the macrophage/granulocyte gelatinase and its presence in plasma. Macrophages and granulocytes are not believed to function in blood but it is possible that these cells secrete the protease during their invasion and phagocytic action in tissues. Then it may leak into plasma where it could be measured by ELISA technique as described recently [19]. While the present results suggest an in vivo role for the macrophage/granulocyte gelatinase and human fibroblast gelatinase/type IV (pro)collagenase, further studies are needed to elucidate their potential significance for instance in the diagnosis or monitoring of pathologic conditions, such as inflammation or malignancies.

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