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Immunopurification and characterization of a collagenase/gelatinase domain issued from basement membrane fibronectin

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Abstract The proteolytic potential of cellular fibronectin fragments issued from a basement membrane hydrolysate was investigated. Three different gelatinase activities (47, 43 and 37 kDa), located by gelatin zymography, were isolated using successively heparin-agarose, gelatin-agarose and immunopurification with polyclonal antibodies directed against bovine plasma fibronectin. These fragments were also characterized using a monoclonal antibody directed against the extra-domain EDA of cellular fibronectin as a probe. A collagenase activity, reliably indicated by the gelatin zymography pattern, was also found using MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2, the intramolecularly quenched fluorogenic substrate of collagenases. From these results, cellular fibronectin was found to be able to exhibit a proteolytic function after limited proteolysis. This MMP-like function could be associated with tissue remodeling in both normal and pathological states, such as metastasis, angiogenesis and tissue repair.

Key words: Cellular fibronectin; Gelatinase; Immunopurification; Monoclonal antibody

1. Introduction

The digestion of basement membranes and extracellular matrices represents a crucial step in tumor spreading and metastasis. Increased levels of proteinases, either intracellular, secreted, or membrane-bound, are associated with the malignant phenotype. Consequently, tumor cell intravasion/extravasion could be dependent on proteinase activities around tumor islets through the digestion of basement membranes [1]. Cysteine proteinases, such as cathepsins B and L, are expressed at higher levels in invasive tumours than in normal or benign tissues and have been implicated in this pericellular proteolysis, in both murine and human cancers [2,3].

In a preceding report [4], we found that cathepsins B and L were able to degrade an intact basement membrane, bovine lens capsule. Fragments from collagen IV, laminin, and fibronectin were solubilized during digestion. On the other hand, gelatinase activities issuing from basement membrane fibronectin were observed [4]. Similar results were obtained using a cysteine proteinase related to cathepsin B, secreted by human adenocarcinomas as an inactive precursor, fully processed in vitro, and then isolated: this latter enzyme was called 'cathepsin B-like' [5].

Plasma fibronectin has been described as a potent proteolytic system activated by limited proteolysis [6,7]. Comparison between both plasma and cellular fibronectin could be very useful to understand some aspects of the molecular mechanisms of tissue remodeling in normal and pathological processes. At the cellular level, the liberation of proteinase activities from basement membrane fibronectin could increase the digestion of collagen IV and laminin fragments liberated by the action of cysteine proteinases and proteinases of other classes [8]. The invasive capacity of tumor cells could be dependent on this complex array of lytic enzymes. Proteinases also modulate tumor angiogenesis through growth factor mobilization and activation [9]. Matrix mettaloproteinases (MMPs) are upregulated during normal and pathological remodeling processes such as tissue repair and tumor invasion [10]. Thus invasion could require the concerted action of several different proteinases [9].

In the present paper, the isolation of these gelatinase activities was performed using successively heparin-agarose, gelatin-agarose and immunopurification with polyclonal antibodies directed against bovine plasma fibronectin. The identification of the fibronectin domains which exhibited the enzymatic activity was carried out using western blotting with a recently described monoclonal antibody directed against the extra-domain EDA of cellular fibronectin [11].

The isolated fragments exhibited an enzymic activity against a intramolecularly quenched fluorogenic substrate of collagenases [12], and the appearance of this enzymic activity was reliable indicated by the time dependency of the zymography pattern observed.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies directed against bovine plasma fibronectin were purchased from UCB-Bioproducts SA (Braine l'alleud, Belgique). Heparin-agarose, gelatin-agarose, CNBr-sepharose, extravidin, biotin peroxidase, anti-mouse biotinylated immunoglobulins and monoclonal antibodies directed against cellular fibronectin (clone FN 3E2) and plasma fibronectin (clone FN 15) were from Sigma (St Quentin Fallavier, France). Immobilon P transfer membranes were from Millipore (Strasbourg, France). The monoclonal antibody directed against the extra-domain EDA of cellular fibronectin was from Biohit S.A. (Bonnelles, France). The fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 was from Nova Biochem (Laufefingen, Switzerland). Fluorescence measurements were carried out on a Kontron SFM 25 spectrofluorometer at λ_{ex} 326 nm and λ_{em} 395 nm using MCA-Pro-Leu for calibration. Digestion experiments were carried out with continuous shaking in a BMAT 25-45 waterbath (Firlabo, Lyon, France).

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Abbreviations: BSA, bovine serum albumin; DTE, dithioerythritol; CNBr, cyanogen bromide; MMP, matrix metalloproteinase; Dpa, *N*-3-(2-4-dinitrophenyl)-L-2,3diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl; EDTA, ethylene diamine tetraacetate, disodium salt

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2.2. Methods

2.2.1. Digestion of bovine lens capsules. Bovine lens capsules were isolated as reported previously [13] and stored at −20°C before use. Incubations were carried out using 100 mM phosphate buffer pH 6.8, containing 1 mM DTE and 2 mM EDTA, without addition of cysteine proteinases, i.e. using only endogenous cathepsin B as digestion proteinase [13]. As reported in the preceding reports [4,13], the incubation times chosen were 24, 48, and 72 h. Other experimental conditions were reported before [4,13]. After digestion, supernatants and digested lens capsules were stored separately at −20°C. The supernatants were blocked with 10 μl of E64 (1.46 mM) before storage.

2.2.2. Affinity chromatography of digestion supernatants on heparin-agarose and gelatin-agarose column. The experimental conditions were identical to those described in [4], and only the 72 h supernatants were used. Bound fractions from both columns, i.e. H^+G^+, were stored at −20°C in 50 mM Tris-HCl buffer, pH 7.5 without $CaCl_2$ for subsequent experiments.

2.2.3. Processing of the gelatinase activities as a function of time. Five samples of 54 μl H^+G^+ or immunopurified fractions were used. Processing was induced by adding 6 μl 100 mM $CaCl_2$ to each sample, giving a 10 mM $CaCl_2$ final concentration, and then the incubation at 37°C was started. Samples were removed and frozen at 0, 24, 48, 72, and 96 h. They were analysed by both zymography and enzymatic activity against the fluorogenic substrate.

2.2.4. Zymography of fractionated supernatants. This was performed using 10% SDS-acrylamide gels containing 1.2 mg/ml bovine skin gelatin as described in [4].

2.2.5. Immunopurification of H^+G^+ fractions. Rabbit polyclonal antibodies directed against bovine plasma fibronectin were covalently linked to CNBr-activated Sepharose 4B using the protocol described in [14]. About 6 mg IgG purified from the whole antiserum as reported in [13] were used for 3 g of CNBr-activated Sepharose 4B. The immunoabsorbant was stored in 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl. The H^+G^+ fraction was applied on the column, and after elution of the unbound material, 200 mM glycine-HCl buffer, pH 2.5 was added to elute the bound proteins. These bound fractions were then re-equilibrated to pH 7.5 using 2 M Tris solution. They were then concentrated and dialysed against the starting buffer.

2.2.6. Determination of the collagenase/gelatinase activities against the fluorogenic substrates. 5 μl of 0.44 mM substrate were mixed with 945 μl of 100 mM Tris-HCl buffer, pH 7.5 containing 100 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35. Incubation was initiated by adding 60 μl of immunopurified fractions processed as described above. The fluorescence of the reaction product was then followed over 2 h. Results were expressed as nM of MCA-Pro-Leu liberated as a function of time through a calibration curve drawn with a 2.25 mM stock solution of this latter peptide as reported [12].

2.2.7. Characterization of the purified fibronectin-gelatinase domain. This was performed using gelatin zymography as described above, dot-blotting with the following antibodies: preimmune sheep polyclonal antibodies, monoclonal antibodies directed against cellular fibronectin (clone FN 3E2) and monoclonal antibodies directed against plasma fibronectin (clone FN15) as reported in [4], and finally by western blotting with monoclonal antibodies directed against the extra-domain EDA of cellular fibronectin using the method described in [4]. Samples of purified material were analyzed after processing at 37°C for 96 h after addition of $CaCl_2$ (10 mM final concentration).

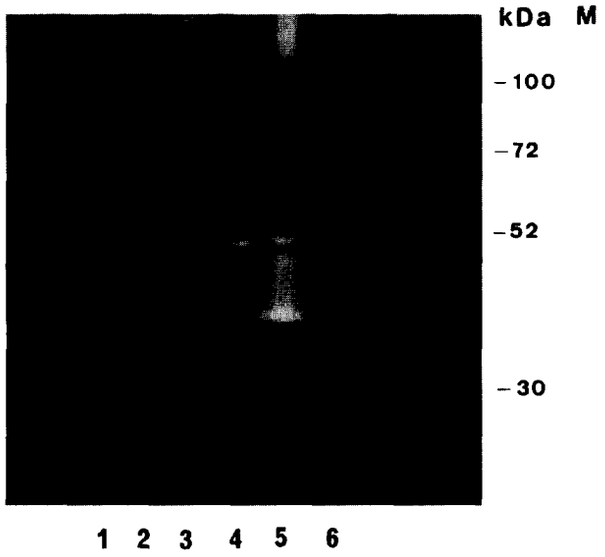

Fig. 1. Gelatin zymography of a basement membrane hydrolysate partially purified on both heparin and gelatin agarose column as a function of time. 54 μl of H^+G^+ fraction were incubated for 24, 48, 72 and 96 h at 37°C together with 6 μl 100 mM $CaCl_2$. The gelatinase activities were vizualized using a 10% SDS-polyacrylamide gel copolymerized with 1.2 mg/ml bovine skin gelatin. Lanes 1–5: samples incubated for 0–96 h at 37°C. Lane 6: molecular mass marker proteins.

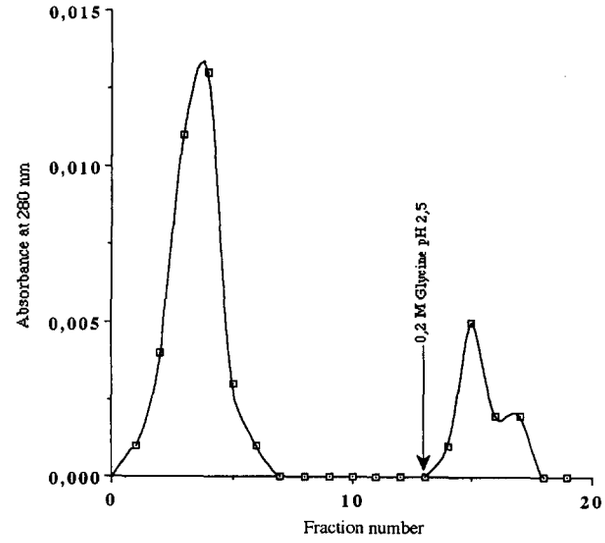

Fig. 2. Elution profile of the H^+G^+ fraction from the polyclonal rabbit plasma antibodies on a CNBr-activated Sepharose column. After processing for 96 h at 37°C with 10 mM $CaCl_2$, the sample was applied on the column equilibated in starting buffer, i.e. 50 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl; after elution of the unbound material, bound proteins were eluted using 200 mM glycine-HCl buffer pH 2.5.

3. Results and discussion

We have characterized 3 gelatinase/collagenase activities stemming from a basement membrane hydrolysate, partially purified by both heparin and gelatin affinity chromatography, using gelatin zymography (Fig. 1). The molecular masses of these activities were 47, 43 and 37 kDa, respectively, and their appearance was dependent on the addition of calcium ions ($CaCl_2$, 10 mM). Under these conditions, by means of gelatin zymography, a time dependency was observed with maximal activity after 96 h incubation at 37°C. The source of these activities was found to be fibronectin, using immunopurification on a column of bovine polyclonal plasma fibronectin antibodies covalently linked to CNBr-activated Sepharose 4B (Fig. 2). Characterization of the bound activity was achieved using gelatin zymography (Fig. 3a) associated with dot-blotting employing 3 different antibodies (data not shown) and western blotting with monoclonal antibodies directed against the extra-domain EDA of cellular fibronectin (Fig. 3b). These gelatinase activities were observed after incu-

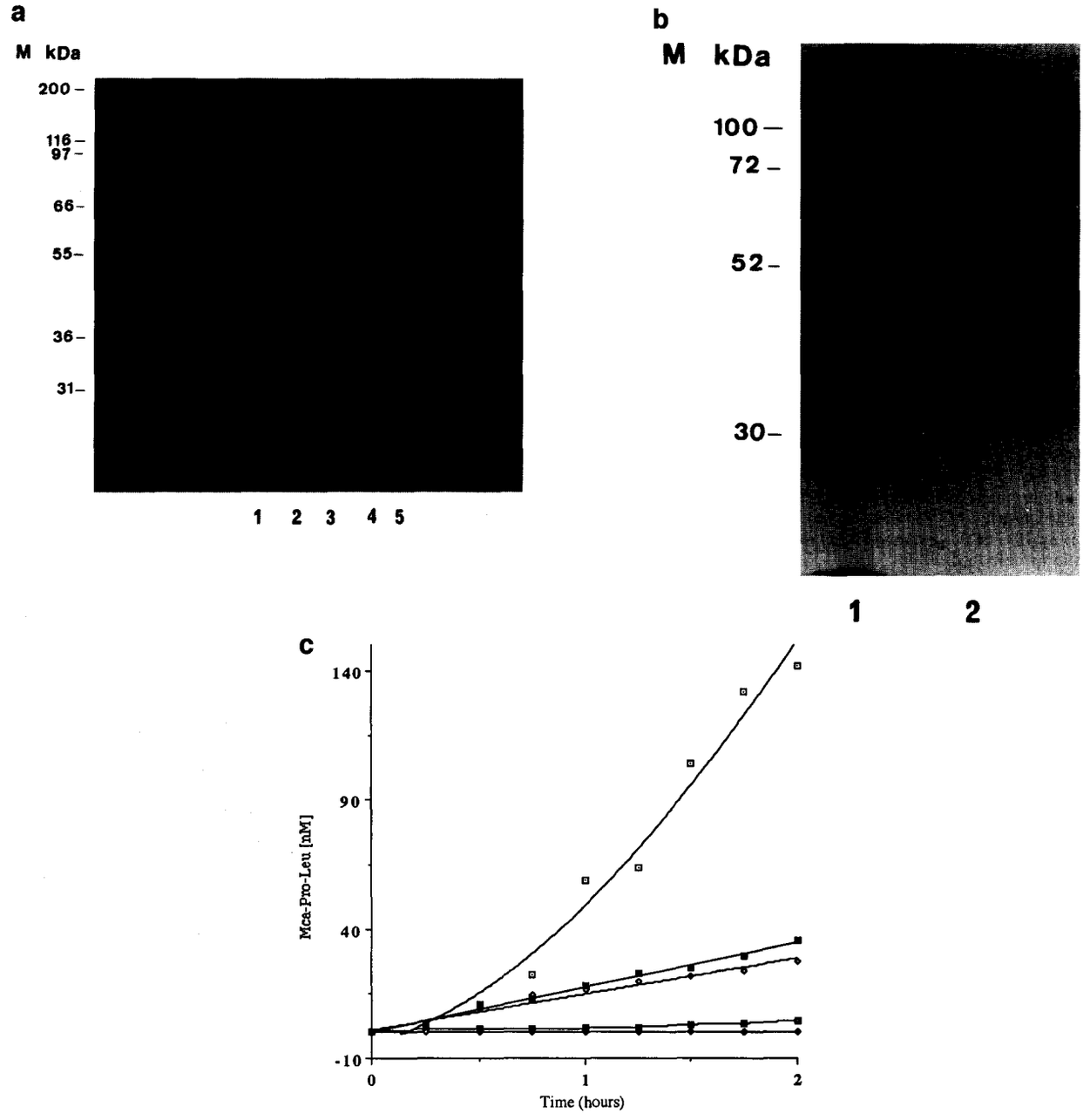

Fig. 3. Characterization of the purified fibronectin-gelatinase fragments. (a) Gelatinase zymography. Lanes: 1, molecular mass marker proteins; 2,4, unbound proteins without incubation (2) or after (4) 96 h incubation at 37°C together with 10 mM $CaCl_2$; 3,5, bound proteins without incubation (3) or after (5) 96 h incubation at 37°C together with 10 mM $CaCl_2$. Experimental conditions were the same as in Fig. 1. (b) Western blotting with monoclonal antibodies directed against the extra-domain EDA of cellular fibronectin. The samples were boiled for 5 min at 95°C with 30 μl of 10% SDS/30% glycerol, followed by 10% SDS-PAGE, and transferred to an Immobilon P membrane sheet (4 h, 300 mA). The sheet was blocked with 2% BSA, 0.2% Brij 35 in PBS overnight. Immunological staining was performed with monoclonal antibodies directed against the extra-domain EDA of cellular fibronectin (1 μg/ml, 1% BSA, 0.2% Brij 35, PBS), anti-mouse biotinylated immunoglobulins (250 μg/ml, 0.2% BSA, 0.2% Brij 35, PBS), extravidine (2 μg/ml, 0.2% BSA, PBS) and biotin horseradish peroxidase (6 μg/ml). The bands were visualized using 4-chloro-1-naphthol. Lane 1: molecular mass marker proteins. Lane 2: isolated fibronectin fragments. (c) Collagenase activity against the fluorogenic substrate as a function of incubation time, at 37°C together with 10 mM $CaCl_2$. Sample incubated for (♦) 0, (□) 24 h, (◇) 48 h, (■) 72 h, (⊡) 96 h.

bation at 37°C for 96 h with 10 mM $CaCl_2$. Collagenase activity was also present in the purified sample, as shown using a fluorogenic substrate of collagenases. For this collagenase activity, a calcium- and time-dependent pattern was found to be similar to the gelatinase activities, i.e. maximal activity was observed after 96 h incubation (Fig. 3c). The proteolytic potential of plasma fibronectin has been investigated in some previous reports [6,7]. However, these properties could be more important at the cellular level [8,13]. To date, it seems that tissue remodeling in both normal and pathological states is dependent on matrix metalloproteinase (MMP) activities. On the other hand, some MMPs such as the 92 and 72 kDa collagenases were found to be structurally related to fibronectin [10,15]. In the present work, we have

shown that after limited digestion of an intact basement membrane, collagenase/gelatinase activities are expressed. The expression of these activities was dependent on both the presence of calcium ions and the incubation time. Nevertheless, the zymographic pattern was not a reliable indicator of those obtained for members of the MMP family: neither 72 nor 92 kDa gelatinase activity was found [16–18], and the gelatinase activities were located within a smaller molecular mass range, i.e. 47, 43 and 37 kDa. On the other hand, the appearance of the enzymic activity was not associated with the addition of organomercurial agents such as *p*-aminophenylmercuric acetate (APMA) (not shown) as reported for the MMPs [10,15]. Therefore, the presence of a 72 kDa gelatinase associated with fibronectin, as reported previously for plasma fibronectin [19], was excluded. The most interesting observation was the immunopurification of both 47, 43 and 37 kDa gelatinase activities by rabbit polyclonal antibodies directed against bovine plasma fibronectin. This result, associated with western blotting using monoclonal antibodies directed against the extra-domain EDA of cellular fibronectin, demonstrates that the source of gelatinase activities was cellular fibronectin. The central region could be the source of the enzymic activities, as been hypothesized in [4]. The central region exhibits a sequence **HHPEH** (1453–1457) related to the consensus sequence **HXXEH** presents in a family of zinc metalloproteinases, the inverzincins. The third zinc ligand could be the **D** or **E** side chain located at position 1531 [20,21]. However, the collagenase/gelatinase activities described here were calcium dependent and this binding site could be related to this cation. Both calcium and zinc ions are required for MMP activities [18], and a small amount of zinc was found associated with MMP samples [22]. In contrast, 5–10 mM calcium ions was usually used for MMPs to exhibit enzymatic activity. Thus, a trace amount of zinc ions present in buffer solutions could activate the proteinase [22] associated with 5–10 mM calcium ions: this fibronectin domain could be structurally related to zinc metalloproteinase families.

Binding of plasma fibronectin to immunoglobulins has been reported elsewhere [23]. In this work, only antibodies directed against cellular fibronectin were bound by dot blotting (not shown) and western blotting. Therefore, this type of non-specific binding was excluded.

On the other hand, fragments issued from plasma fibronectin were found to be associated to cartilage chondrolysis by increasing the biosynthesis of several MMPs such as stromelysin 1 [24]. Consequently, fibronectin domains could be directly involved in proteolysis and tissue remodeling, and they could also increase the level of several MMPs through gene overexpression [25]. As a working hypothesis, we suppose that both functions are connected and are components of the same regulation pathway. Biological activities not detected in intact fibronectin could be associated with proteolytic fragmentation of fibronectin. Alternatively, these activities may be exposed through a conformational change. Some functional domains are buried in the fibronectin structure and can be exposed by such treatments [26]. Both modifications, i.e. limited proteolysis and conformational change, could be involved in the observations reported in the present paper.

In a recent report, it has been shown that gelatinase A (72 kDa gelatinase) is associated with cellular events required for effective invasion, such as cell matrix attachment, detachment and migration [25]. Binding of progelatinase A to the extracellular matrix produced by bovine corneal endothelial cells has been reported [27], confirming these biological roles. Gelatinase A contains a 19 kDa fibronectin-like insert in the catalytic domain. When this insert was deleted by construction of a synthetic gene, the resulting enzyme showed the same activity pattern as the full length proteinase, excepted a reduction in catalytic efficiency against gelatin, suggesting and involvement of the fibronectin-like domain, which is a gelatin binding domain, in catalysis towards protein substrates [22]. Taken together, these results underline the link between the MMPs and fibronectin, and suggest that fibronectin is a multidomain adhesion protein which could exhibit different properties. Nevertheless, little is known on the in vivo functioning of these domains in the intact protein [28]; they could be buried and therefore inactive [26]. However, when fibronectin domains are separated by limited proteolysis, other properties are expressed, especially a MMP-like function, as reported here. The expression of a MMP-like activity from cellular fibronectin fragments provides new insights into cellular fibronectin functioning with regard to tumor cell invasion and tissue remodeling. To date, synthetic peptide-based assays can be developed to discriminate between the different MMPs, using the new fluorogenic substrates recently described [29]. In further work, this method together with N-terminal sequencing of purified fragments should provide a molecular definition of the collagenase/gelatinase activities issued from basement membrane fibronectin.

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