Volume 314, number 3, 386–388 FEBS 11904 December 1992

A 25 kDa α_2-microglobulin-related protein is a component of the 125 kDa form of human gelatinase

Susanne Triebel, Jörg Bläser, Heinz Reinke and Harald Tschesche

Department of Biochemistry, Faculty of Chemistry, University of Bielefeld, W-4800 Bielefeld 1, Germany

Received 14 October 1992

Besides the monomeric mammalian 95 kDa progelatinase, two additional forms, a disulfide-bridged 220 kDa dimer and a 125 kDa form were isolated from human PMN leukocytes. The 125 kDa progelatinase was identified as a covalently linked, disulfide-bridged heterodimer formed of the monomer with a 25 kDa protein. This 25 kDa protein was isolated from gelatinase bound to the affinity support of gelatin–Sepharose and eluted by DTE-containing buffer. The amino acid sequence of tryptic peptides of this protein revealed homology with an α_2-microglobulin-related protein from rats, a protein so far unknown in humans.

Gelatinase; α_2-Microglobulin-related protein; Metalloproteinase; Lipocalin

1. INTRODUCTION

95 kDa gelatinase, or matrix metalloproteinase-9 (MMP-9), is a neutral metalloproteinase which has been isolated from neutrophils, macrophages and transformed fibroblasts. Gelatinase degrades a number of matrix components, including elastin [1], cartilage proteoglycan [1], collagen types IV [2], V [3], XI [1] and type I gelatin.

MMP-9 has the most complex domain structure of all members of the matrix metalloproteinase family. In addition to the signal peptide (not in the mature protein), the propeptide, the catalytic and the C-terminal hemopexin-like domain are a fibronectin-homologous region inserted into the catalytic domain, and a type V collagen-homologous region inserted in the C-terminal domain [4]. The fibronectin-like domain can bind gelatin [5], a fact with practical application for the purification of gelatinase, but without relevance for substrate specifity [6]. The type V collagen-like domain contains 1 cysteine residue, which is assumed to participate in the dimerization of gelatinase [2]. A special feature of MMP-9 isolated from neutrophils is that it exists partially in higher molecular weight forms. Neutrophils secrete the monomeric 95 kDa form, a reduction-sensitive 220 kDa homodimer and 125 kDa heterodimer. Here we report on the isolation of the disulfide-linked 25 kDa protein from the heterodimer. Determination of parts of the amino acid sequence revealed homology to an α_2-microglobulin-related protein from rats, so far not discovered in humans.

Correspondence address: H. Tschesche, Universität Bielefeld, Fakultät für Chemie, Abteilung Biochemie, Postfach 100131, W-4800 Bielefeld 1, Germany. Fax: (49) (521) 106 6146.

Abbreviations: PMNL, polymorphonuclear leukocytes; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; DTE, dithioerythritol; FPLC, fast performance liquid chromatography; HPLC, high performance liquid chromatography.

2. MATERIALS AND METHODS

Endoglycosidase *F*/*N*-glycosidase F was purchased from Boehringer-Mannheim Biochemica (Germany) and TPCK-treated trypsin was from Merck (Darmstadt, Germany). Plasmatonin was a gift from Fresenius (Oberursel, Germany).

2.1. *Isolation and purification of PMNL progelatinase*

PMNL progelatinase was isolated as published elsewhere [7] as a mixture of three molecular mass forms (220, 125 and 95 kDa). Separation of free progelatinase and progelatinase–TIMP-1 complex was achieved by heparin–Sepharose chromatography as described by Kolkenbrock [8] for the 72 kDa gelatinase and its complex with TIMP-2.

2.2. *Separation of the 25 kDa protein*

TIMP-1-free progelatinase was adsorbed to a gelatin–Sepharose column. Elution with buffer A (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 5 mM $CaCl_2$, 0.02% NaN_3) containing 10 mM DTE, yielded the reduced 25 kDa protein. The 98 kDa gelatinase was eluted with buffer A containing 2.5% DMSO.

2.3. *Carboxymethylation of the 25 kDa protein*

10 mg gelatinase mixture was dialysed against 0.1 N acetic acid, lyophilized and dissolved in 2.5 ml buffer B (0.5 M Tris-HCl, pH 8.1, 6 M guanidine hydrochloride, 2 mM EDTA). The solution was incubated under argon atmosphere for 30 min at 50°C. Then 5 mg DTE was added under argon atmosphere and the solution was incubated for a further 3 h at 50°C. 13 mg iodoacetamide dissolved in buffer B were added at room temperature and incubated in the dark for 30 min. The reaction mixture was directly subjected to an FPLC gel-filtration column yielding pure carboxymethylated 25 kDa protein and the carboxymethylated progelatinase.

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2.4. *Characterisation and identification of the 25 kDa protein*

2.4.1. Deglycosylation with endoglycosidase F

1,000 μl carboxamidomethylated 25 kDa protein were lyophilized and dissolved in buffer C (100 mM $K_3PO_4 \cdot H_2O$, pH 6.5, 50 mM EDTA, 1% Triton X-100). After 5 min heating at 100 °C 1 μl endoglycosidase F was added and incubated for 2 days at 37°C.

2.4.2. Cleavage by trypsin

1.5 ml 25 kDa protein was dialyzed against buffer D (20 mM Tris-HCl, pH 8, 1 M NaCl) and incubated overnight with 1 μl TPCK-treated trypsin. The reaction mixture was concentrated and directly subjected to HPLC on a Bakerbond C_{18} wide-pore reverse-phase column. The peptides were isolated at a constant flow rate of 0.8 ml/min using a linear gradient of 0–80% acetonitrile, and further subjected to automated amino acid sequence determination.

3. RESULTS AND DISCUSSION

Analysis of the gelatinase preparation from PMN leukocytes by SDS-PAGE showed 3 protein bands with apparent molecular weights of 220, 125 and 95 kDa [6]. The N-termini of the different gelatinase forms were found to be identical and were the same as that of the proenzyme from SV-40-transformed lung fibroblasts [2]. All 3 forms showed no activity against the synthetic DNP octapeptide prior to activation. Under reducing conditions in SDS-PAGE these forms resulted in a 95 and 25 kDa protein band [6]. Consequently the 25 kDa protein could be isolated from progelatinase bound to gelatin–Sepharose by reducing buffer. The N-terminus of this protein was blocked against Edman degradation. The isolation of tryptic peptides of the carboxymethylated 25 kDa protein allowed the the first sequence information to be obtained. Comparison of the sequence data with that of proteins of the EMBL protein database revealed homology to an α_2-microglobulin-related protein from rats [9] (Fig. 1). Homology to this family of proteins was supported by similarity in molecular weights. Deglycosylation by endoglycosidase F reduced the molecular weight of the human 25 kDa glycoprotein by removal of the *N*-glycosidic-bound oligosaccharides to 22 kDa (Fig. 2). This compared well to the 22.6 kDa estimated from the cDNA for the rat protein.

The secretion of high molecular weight gelatinase forms is a special feature of PMN leukocytes. Macrophages [10] and transformed cell lines [2] produce only the monomeric form. The physiological roles of the homodimer and the heterodimer are still unknown. Recently, a 130 kDa gelatinolytic enzyme has been found to participate in involution of the mammary gland of rats [11]. Besides this, Backstrom et al. [12] identified a gelatinolytic enzyme with the same molecular weight in Alzheimer-affected hippocampus tissue of humans. Our characterization of the heterodimer may facilitate the determination of its physiological role. The protein to which the 25 kDa protein is homologous has been characterized, in so far that the mRNA amount in the liver and prostate of male rats is the same, but lower in the

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1
2 MGLGVLCLAL VLLGVLQRQA QDSTQNLIPA PPLISVPLQP GFWTERFQGR
3                                    EEASSTRG NLDVAKLNGD
4                                GPVP TPPDNIQVQE NFNISRIYGK

1 NYVVGLAGNA I           MYATIYELKE DNSYNVTS?L
2 NFVVGLAANA VQKERQSRFT MYSTIYELQE DNSYNVTSIL VRGQGCRYWI
3 WFSIVVASNK REKIEENGSM RVFMQHIDVL ENSLGFKFRI KENGECRELY
4 WYNLAIGSTC PWLKKIMDRM TVSTLVLGEG ATEAEISMTS TRWRGVCEET

1                                                    S?N?
2 RTFVPSSRPG QFTLGNIHSY PQIQSYDVQV ADTDYDQFAM VFFQKTSENK
3 LVAYKTPEDG EYFVEYDGGN T          FTI LKTDYDRYVM FHLINFKNGE
4 SGAYEKTDTD GKFLYHKSKW NITME      SYV VHTNYDEYAI FLTKKFSRHH

1 ?YFKVTLYGR                    SLG LPENHIVFPV PIDQ??P
2 QYFKVTLYGR TKGLSDELKE RFVSFAKSLG LKDNNIVFSV PTDQCIDN
3 TFQLMVLYGR TKDLSSDIKE LFAKLCEAHG ITRDNIIDLT KTDRCLQARG
4 GPTITAKLYG RAPQLRETLL QDFRVVAQGV GIPEDSIFTM ADRGECVPGE

1
2
3
4 QEPEPILIPR
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Fig. 1. Comparison of the amino acid sequences of (1) α_2-microglobulin-related protein from humans identified as a component of the 125 kDa form of gelatinase, (2) α_2-microglobulin-related protein from rats [14], (3) α_2-microglobulin from rats [14], (4) α_1-microglobulin from humans [14]. The underlined regions indicate those sequences used to identify homologies within the family of lipocalins.

liver of females [8]. The question of whether the PMN leukocytes of male and female humans contain the same amounts of the 125 kDa gelatinase will be the subject of future investigations. The protein to which the rat protein is related, α_2-microglobulin, is the major urinary protein of sexually mature rats, but it is not expressed by humans [13]. It is produced in the liver and involved in hyaline droplet nephropathy. Its exact role in normal physiology is not understood at present.

On the basis of amino acid sequence homology, α_2-microglobulin has been allocated to the group of pro-

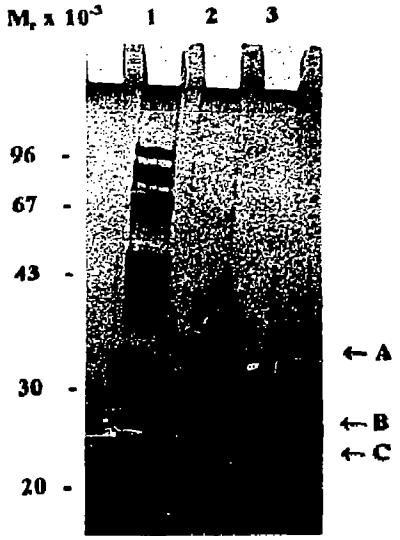

Fig. 2. Demonstration of the molecular weights before and after deglycosylation (10% SDS-PAGE, silver stained). Lane 1, marker proteins; lane 2, endoglycosidase F (A), glycosylated α_2-microglobulin-related protein (B), deglycosylated α_2-microglobulin-related protein (C); lane 3, α_2-microglobulin-related protein without endoglycosidase.

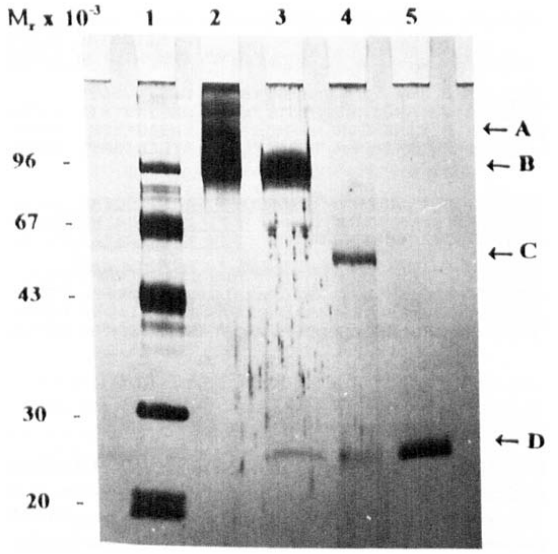

Fig. 3. Isolation of the α_2-microglobulin-related protein (10% SDS-PAGE, silver stained). Lane 1, marker proteins; lane 2, different gelatinase forms, (A) 125 kDa, (B) 95 kDa monomer; lane 3, different gelatinase forms under reducing conditions, (D) 95 kDa monomer and α_2-microglobulin-related protein; lane 4, α_2-microglobulin-related protein after gelatin–Sepharose and dialysis against non-reducing buffer, dimer (C) and monomer (D); lane 5, carboxymethylated α_2-microglobulin-related protein (D).

teins called lipocalins [14]. This protein family includes proteins such as retinol binding protein [15], α_1-acid glycoprotein [16], complement C8γ [17] and α_1-microglobulin [18], all identified in human urine or serum. The common feature of all lipocalins is their capacity to transport small lipophilic ligands through the hydrophilic body fluid [13]. Some members, based on their odd cysteine number, could be isolated as disulfide-linked heterodimers with other proteins. In humans, α_1-microglobulin was found as a heterodimer with IgA [17] and C8γ bound to C8α in human complement C8 [16].

Fig. 3 shows the lipocalin protein isolated by us bound to neutrophil gelatinase. The free form isolated under reducing conditions dimerized during oxidation. Disulfide-linked homodimers and heterodimers are still unknown in the other members of the matrix metalloproteinase family. All have two conserved cysteine residues in the hemopexin-like domain, which are assumed to be disulfide bridged [14]. Recently we found that these cysteines and one additional cysteine in this domain of PMNL gelatinase are not disulfide linked [19]. This may be the basis of homodimer and heterodimer formation.

The physiological significance of the connection between the metalloproteinase and the lipocalin families established by a gelatinase α_2-microglobulin-related protein heterodimer will be the subject of further investigations. The lipocalin protein showed neither inhibitor nor activator activity against gelatinase, as determined by degradation of synthetic DNP octapeptide. Some lipocalin proteins are also known to transport retinol through the hydrophilic body fluid [13]. Furthermore, it has been shown recently [20–22] that retinoic acid can modulate the secretion of TIMP and the metalloproteinases. Whether the α_2-microglobulin-related protein can participate in similar processes remains unknown at present. α_1-Microglobulin, a closely related protein, has been found to affect the chemotaxis of neutrophils [23]. It will soon be shown whether the new lipocalin member has any effect on human neutrophils.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, special research programme SFB 223, project B2. The authors wish to thank R. Beckmann for his assistance with the computer research and G. Delany for linguistic advice.

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