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Evidence for the involvement of type II domains in collagen binding by 72 kDa type IV procollagenase

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The fibronectin-related region of the 72 kDa type IV procollagenase has been expressed in *E. coli* as a β-galactosidase fusion product. The fragment containing the three type II units of the protein was found to have affinity for denatured collagen, suggesting that these domains may be responsible for the collagen-affinity of type IV collagenase. We have also shown that segment Ala-Ala-His-Glu of type IV collagenase (residues 372–375), which is similar to a fibronectin-segment previously implicated in collagen-binding, is not essential for binding activity.

Type IV collagenase; Fibronectin; Matrix degradation; Tissue remodelling; Tumor invasion

1. INTRODUCTION

Fibronectin is a large extracellular protein made up of multiple copies of three types of homology units [1]. Segments homologous with the so called type II domains of fibronectin have been identified in a variety of proteins such as insulin-like growth factor II receptor/mannose 6-phosphate receptor [2,3], mannose receptor [4], blood clotting factor XII [5], bovine seminal fluid proteins PDC-109 and BSP A3 [6,7], 72 kDa and 92 kDa type IV collagenases [8,9]. Since these proteins do not appear to share a common binding specificity it seems likely that their type II domains may have different roles. Recently we have shown that isolated type II domains of fibronectin and PDC-109 are capable of binding denatured collagen as well as hydrophobic ligands [10]. However, no information is available on the binding specificity of type II domains of other proteins.

Type IV collagenases play a key role in matrix remodelling, degradation of basement membranes and contribute to the metastatic potential of tumor cells [11]. These enzymes are unique among mammalian metalloproteinases in displaying marked affinity for gelatin and collagens of the basement membrane. In the present work we show that the type II domains of the 72 kDa type IV procollagenase have specific affinity for gelatin-Sepharose 4B indicating that these domains are responsible for the collagen affinity of the protein.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were purchased from BRL (Bethesda, MD, USA) and Biolabs (Beverly, MA, USA). Oligodeoxyribonucleotides were synthesized on a CRB GENESIS DNA synthesizer (Cambridge Research Biochemicals, Cambridge, UK) with the phosphotriester method and purified on MonoQ and MonoRPC columns using an LKB HPLC.

The M13 sequencing kit used for dideoxy sequencing of cloned DNA fragments was from Amersham International, the Site Directed Mutagenesis Kit was obtained from Boehringer (Mannheim, Germany). Gelatin Sepharose 4B (Pharmacia) according to the instructions of the manufacturer.

2.2. *Plasmids*

Plasmid pBST4coll, the Bluescript vector containing the cDNA insert of pGel 186.2 of 72 kDa type IV collagenase in its *Sma*I site [8] was obtained from Dr. G.I. Goldberg (Washington University, St. Louis, USA). The *E. coli* expression plasmid pmed23 [12] was obtained from Dr. P. Venetianer (Biological Research Center, Szeged, Hungary).

2.3. *Construction of plasmid expressing the three type II domains of type IV collagenase*

pBST4coll was digested with *Eco*RI and *Bgl*II and the 1.4 kb fragment corresponding to the N-terminal part of type IV collagenase (but lacking most of the hemopexin-like domain) was isolated by electrophoresis on 1% low melting agarose using the protocol of Qiagen, Studio City, CA). The fragment was cloned into the *Eco*RI/*Bam*HI site of M13 mp18 sequencing vector and the replicative form of the recombinant M13 mp18-G/6 was digested with *Ava*II and *Bal*I and the 558 bp fragment (nucleotides 613–1170 of the cDNA coding for the three type II units) was isolated (Fig. 1). The fragment was blunt-ended by filling in with DNA polymerase I Klenow fragment and was cloned into the *Sma*I site of M13 mp19. In recombinant clones (M13 mp 19/123) the insert was found in both orientations.

Single stranded DNA of M13 mp19/123 was mutagenized (Fig. 2) with the mutagenic oligodeoxyribonucleotide 5′GAACAGGCTTTA-CCCTTGGT3′ according to the gapped-duplex method [13] using a Site Directed Mutagenesis Kit (Boehringer, Mannheim, Germany). Mutant phages were identified by plaque hybridization and the mutation was verified by sequencing with the dideoxy chain termination

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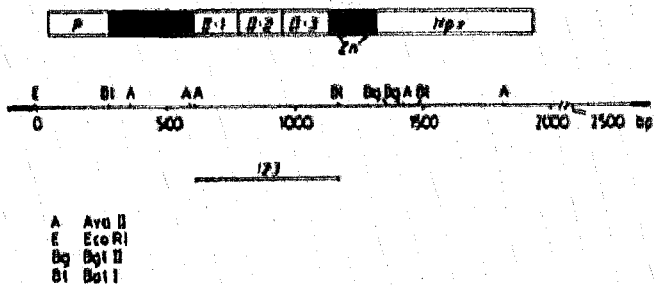

Fig. 1. cDNA fragment of human 72 kDa type IV procollagenase expressed in *E. coli*. The figure shows the structure of the 72 kDa type IV procollagenase; the shaded block represents the metalloprotease domain, P indicates the propeptide domain of the proenzyme, Zn indicates the zinc-binding site, Hpx represents the hemopexin-like domain and II-1, II-2, II-3 mark the three fibronectin-homologous type II domains. The position and size of the cDNA fragment expressed (fragment 123) and the positions of the restriction sites of pBST4coll used in the construction of the expression plasmids are also shown. In the scheme of pBST4coll the cDNA insert is indicated with a thin line.

method [14]. The replicative form of mutagenized M13 mp19/123 was digested with *Bam*HI, blunt-ended then was digested with *Eco*RI and ligated with the *Pvu*II/*Eco*RI-digested expression vector pmed23. By cloning the insert into the *Pvu*II site of the vector the β-galactosidase portion of the fusion protein is reduced to 37 residues [12].

2.4. *Bacterial expression*

Escherichia coli cells carrying recombinant pmed23 plasmids were grown, expression of β-galactosidase fusion proteins was induced with 100 μM IPTG and fusion products were isolated essentially as described previously [10]. Briefly, the IPTG-induced cells were harvested by centrifugation at 4°C and the pellet was washed with ice cold 25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0 and centrifuged at 4°C. The pellet was suspended in the same buffer containing 1 mg/ml of lysozyme and incubated at 0°C for 10 min. The cells were disrupted with an MSE ultrasonicator, the inclusion bodies were collected by centrifugation and then washed with the above buffer. The pellet containing the inclusion bodies was dissolved in 0.1 M Tris-HCl, 6 M guanidinium chloride buffer, pH 8.0 containing 100 mM dithiothreitol and was incubated at 25°C for 1 h with constant stirring. Insoluble cellular debris were removed by centrifugation and the solution was gel-filtered on a Sephadex G-25 column equilibrated with 0.1 M Tris-HCl, 3 M urea, 5 mM EDTA pH 8.0 to remove the reducing agent. The fractions containing the protein were pooled, and

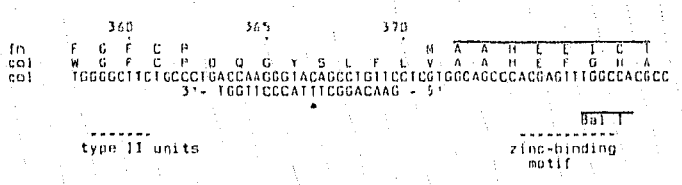

Fig. 2. Introduction of a stop codon at the boundary separating the third type II domain from the zinc-binding motif of 72 kDa type IV collagenase. The amino acid sequence [1] of the pertinent region of human fibronectin (fn), the amino acid and nucleotide sequences [8] of human 72 kDa type IV procollagenase (col) and the sequence of the mutagenic primer are shown; the site of mutagenesis is marked by an asterisk. The *Bal*I site used in the construction of expression vector pmed23/123 is also shown. The first 8 residues of the segment implicated in collagen binding by fibronectin [16] are overlined. The numbers refer to the amino acid sequence of human 72 kDa type IV procollagenase.

then reduced (10 mM) and oxidized glutathione (1 mM) were added. The solution was incubated at 25°C for 24 h then dialyzed against 0.1 M ammonium bicarbonate, pH 8.0. The small amount of precipitated protein was removed by centrifugation and the protein was lyophilized.

2.5. *Other techniques*

The collagen affinity of the β-galactosidase fusion protein containing the three type II domains of type IV collagenase (β-gal coll 123) was assayed by affinity chromatography on gelatin-Sepharose 4B columns (HR10/30) using an LKB HPLC as described previously [10].

SDS-PAGE was performed according to a described procedure [15], proteins were stained with Coomassie brilliant blue G-250 and the molecular weights were estimated using a Pharmacia low molecular weight calibration kit.

3. RESULTS AND DISCUSSION

The fibronectin-homolog region of human 72 kDa type IV procollagenase has been expressed in *E. coli* as a β-galactosidase fusion product (β-gal coll 123), isolated from inclusion bodies and refolded essentially as described for the type II domains of fibronectin [10]. The fusion protein consists of residues 1–37 of β-galactosidase and residues 194–345 of the 72 kDa type IV collagenase. The calculated molecular mass of the protein is 23 370 Da.

We have found that using our refolding protocol [10] a significant portion (80–90%) of the fusion protein was bound to gelatin-Sepharose 4B (Fig. 3A). When bound protein was desalted and reapplied onto a gelatin-Sepharose 4B column complete rebinding was observed (Fig. 3B), supporting the specificity of the interaction. Furthermore, binding of the protein to gelatin-Sepharose 4B was shown to be specific for gelatin since no binding was observed when the protein was loaded onto unsubstituted Sepharose 4B (Fig. 3C).

The present data thus provide experimental support for the earlier suggestion that the fibronectin-like region of type IV collagenases might be responsible for the affinity of these metalloproteinases for gelatin [8,9]. This suggestion was based on the fact that the homologous region is part of the collagen-binding domain of fibronectin [1,8,9].

Previous studies on fibronectin suggested that a 14-residue segment C-terminal to its second type II domain is critical for collagen binding [16]. As noted by Collier et al. [8], in this region a stretch of 4 residues (AAHE in Fig. 2) are shared by fibronectin and type IV collagenase.

Some considerations, however, argue against a role of the AAHE sequence in collagen binding by type IV collagenases. First, a survey of the sequences of mammalian metalloproteases [8,9] reveals that AAHE is present in all mammalian metalloproteases although only type IV procollagenases have affinity for gelatin. Second, AAHE is part of the conserved Zn^{2+}-binding site essential for catalysis by metalloproteases thus its involvement in gelatin binding would seem unlikely. Third, the exon-intron structure of the gene of type IV

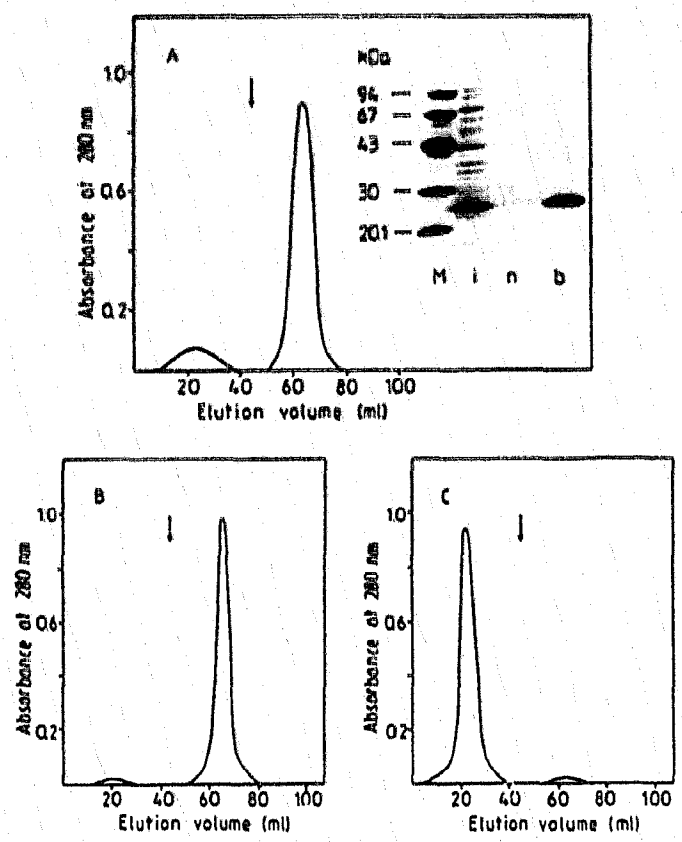

Fig. 3. (A) Affinity chromatography of fusion protein β-gal coll 123 on gelatin-Sepharose 4B. The fusion protein was isolated and refolded as described in the text. The protein was applied onto gelatin-Sepharose 4B columns (HR 10/30) equilibrated with 100 mM ammonium bicarbonate buffer, pH 8.0 and the column was washed with the same buffer; the bound proteins were eluted with buffer containing 8 M urea. The arrow marks the start of elution with buffer containing 8 M urea. The inset shows the SDS-PAGE patterns of the proteins of IPTG-induced bacteria (i), protein not bound (n) and bound (b) to gelatin-Sepharose 4B and molecular mass markers (M). The numbers indicate the molecular masses of marker proteins. (B) Rechromatography of fusion protein β-gal coll 123 on gelatin-Sepharose 4B. The bound protein from the first affinity chromatography step (Fig. 3A) was desalted in 100 mM ammonium bicarbonate, pH 8.0, reapplied onto a gelatin-Sepharose 4B column (HR 10/30) equilibrated with 10 mM ammonium bicarbonate buffer, pH 8.0 and the column was washed with the same buffer. The arrow marks the start of elution with buffer containing 8 M urea. (C) Chromatography of fusion protein β-gal coll 123 on Sepharose 4B. The fusion protein in 100 mM ammonium bicarbonate, pH 8.0 was applied onto Sepharose 4B columns (HR 10/30) equilibrated with 100 mM ammonium bicarbonate buffer, pH 8.0 and was washed with the same buffer. The arrow marks the start of elution with buffer containing 8 M urea.

collagenase clearly reveals that the AAHE sequence derives from the ancestral metalloprotease and not from the fibronectin-like ancestor [17] thus the presence of this tetrapeptide sequence in both type IV collagenase and fibronectin appears to be a coincidence. Although these arguments do not disprove the involvement of AAHE in collagen binding they raise qeustions as to what specific part of type IV collagenase is involved in binding.

In the present work we have introduced a stop codon between the third type II domain and the AAHE sequence (Fig. 2). The fact that the fusion protein possessing the three type II units but lacking the AAHE sequence binds to gelatin-Sepharose 4B proves that the latter sequence is not essential for gelatin affinity and indicates that type II units play the major role in collagen binding. This conclusion also harmonizes with our previous findings on collagen binding by isolated type II units of bovine seminal fluid protein PDC-109 and fibronectin [10].

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