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# The gelatin-binding site of human 72 kDa type IV collagenase (gelatinase A)

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To identify structures critical for gelatin-binding of 72 kDa type IV collagenase (gelatinase A), fragments of this metalloproteinase have been expressed in *Escherichia coli* and assayed for their gelatin affinity. Each of the three fibronectin-related type II domains was found to have affinity for gelatin. Fragments containing all three tandem type II domains had significantly stronger affinity than any of the constituent units, indicating that they co-operate to form the high-affinity gelatin-binding site. Competition experiments have also shown that gelatinase A binds more tightly to gelatin than fibronectin and can displace the latter from denatured collagen.

## INTRODUCTION

Proteolytic degradation of constituents of the extracellular matrix and basement membranes plays an important role in tissue-restructuring processes such as those accompanying cell migration, morphogenesis, wound healing, angiogenesis and tumour invasion [1]. The proteinases implicated in tumour invasion include components of the urokinase receptor/urokinase/plasminogen system and several members of the metalloproteinase family [2–7]. Enzymes capable of degrading type IV collagen are thought to be especially important in tumour metastasis as this collagen is a major component of basement membranes that have to be penetrated during migration of tumour cells. In harmony with this notion, secretion of type IV collagenases was found to be well correlated with metastasis and transformation [1,4–6,8].

Studies on the primary structures of 72, 92 and 105 kDa gelatinases have revealed that they contain a catalytic domain, a haemopexin-like domain and, unlike other members of the metalloproteinase family, three tandem homology units closely related to the type II domains of fibronectin [9–11].

The haemopexin-like domain of gelatinase A has been shown to be involved in modulation of its activity by the tissue inhibitors of metalloproteinases (TIMPs) [12–14]. This region is also required for the activation of the 72 kDa type IV procollagenase (progelatinase A) by a membrane-associated activator, suggesting that this domain of the proenzyme may interact with some component of the cell membrane [13].

Studies on the substrate specificity of gelatinase A and B have shown that they are able to degrade type IV, type V, type VII and type X collagens, fibronectin, elastin and all types of denatured collagens [8,15,16]. Progelatinases are unique among metalloproteinases in having pronounced affinity for gelatin, permitting their isolation by affinity chromatography on gelatin–Sepharose [17]. It was shown recently that the gelatin-binding sites of gelatinases A and B reside in their fibronectin-related regions [18,19].

The biological significance of the marked gelatin-affinity of gelatinases is not fully understood. We have initiated a structure–function study of the gelatin-binding domain of gelatinase A with a view to defining its ligand specificity and function. As a first step, we wished to identify more precisely the structures critical for gelatin binding. Here, we report the expression of type II domains of the fibronectin-related region of the gelatinase A in *Escherichia coli* and the characterization of their gelatin-binding properties. Our results indicate that each of the three type II domains contribute to the gelatin affinity of an extended, high-affinity gelatin-binding site.

## MATERIALS AND METHODS

Plasmid pBST4coll containing the cDNA of human gelatinase A [9] was obtained from Dr. G. I. Goldberg (Washington University, St. Louis, MO, U.S.A.); expression plasmid pmed23 [20] was from Dr. P. Venetianer (Biological Research Center, Szeged, Hungary). *E. coli* strain JM-109 was used to propagate and amplify expression plasmids.

Gelatin–Sepharose 4B was prepared using porcine skin type I collagen (Sigma) and cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. The 42 kDa gelatin-binding fragment of human fibronectin was isolated as described [21].

The plasmid expressing the three tandem type II domains of gelatinase A was constructed from pmed23 as described previously [18]. Plasmids expressing different type II domains of gelatinase A were obtained by similar procedures, by exploiting restriction sites located at the boundaries of type II units (Figure 1). An additional *Nco*I site was introduced between the second and third type II units, at nucleotides 960–965, by mutagenesis with the oligonucleotide 5′-pCCAACAGTGGCCATGGCG-GT-3′ according to the gap duplex method [22]. All recombinant proteins contain a 37-amino-acid long N-terminal part derived from the β-galactosidase moiety of the pmed23 expression vector [20]. The combinations of type II domains present in the different recombinant proteins are shown in Figure 1.

*E. coli* JM-109 cells carrying recombinant pmed23-derived plasmids were grown, expression of β-galactosidase fusion proteins was induced with 100 μM isopropyl thiogalactoside, and fusion products were isolated from inclusion bodies and refolded as described previously [18,23]. Recombinant proteins were applied to gelatin–Sepharose 4B column equilibrated with 0.1 M Tris/HCl, 10 mM EDTA, pH 8.0, and the bound proteins were eluted with 8 M urea in the same buffer. In the affinity chromatography experiments, the samples exhausted less than 20% of the column capacity. The bound proteins were subjected to a second step of affinity chromatography on gelatin–Sepharose 4B,

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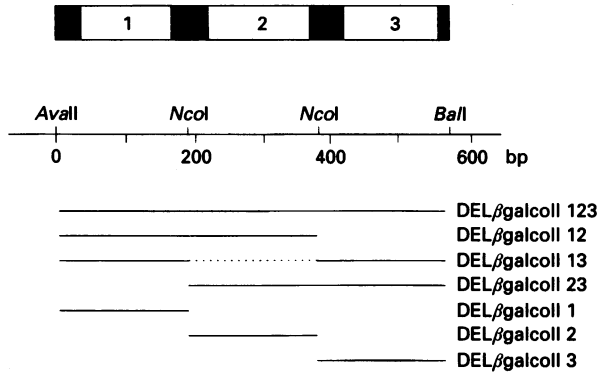


**Figure 1 Schematic representation of the gelatin-binding domain of human gelatinase A and the cDNA fragments expressed in *E. coli***

The figure shows the three type II domains (blocks marked 1, 2 and 3), the position of the cDNA fragments expressed and the positions of the restriction sites used in the construction of the expression plasmids. The *NcoI* cleavage site between the second and third type II units was introduced by site-directed mutagenesis. The restriction sites used in the construction of vectors expressing fragments of the gelatin-binding domain are located at boundaries of type II domains; thus in each case, intact type II domains are expressed.

using a 0–8 M urea gradient for elution of bound proteins (see Figure 3).

Affinity of recombinant proteins for immobilized gelatin was studied by mixing aliquots of gelatin–Sepharose 4B (100 $\mu$l) with 1 ml of 0.1 M Tris/HCl, 10 mM EDTA, pH 8.0, buffer containing different concentrations of recombinant proteins. The mixtures were stirred for 16 h at 25 °C, and then centrifuged. The concentration of the protein in the supernatant (free protein) was determined spectrophotometrically using molar absorption coefficients determined as described [24]. The concentration of protein bound to gelatin–Sepharose 4B was calculated by subtracting the concentration of the free protein from the total concentration of the protein. The data obtained in these experiments are shown in Figure 4. Apparent association constants were calculated from half-maximal saturation points.

The competition of different fragments of gelatinase A for gelatin was studied as described previously [25,26]. In these competition assays, proteins recognizing the same or overlapping sites of gelatin may be expected to compete as a function of their relative gelatin affinities according to the equation: $K_i/K_1 = ([LP_i]/[LP_1]) \times ([P_1]/P_i])$ where $[LP_i]/[LP_1]$ is the ratio of the concentrations of the bound proteins, $[P_1]/[P_i]$ is the ratio of the concentration of the two proteins in equilibrium with gelatin-Sepharose, and $K_i/K_1$ is the ratio of the apparent association constants of the interaction of the two proteins with gelatin–Sepharose. In competition experiments, 100 $\mu$l of gelatin–Sepharose 4B was added to 1 ml of 0.1 M Tris/HCl, 10 mM EDTA, pH 8.0, buffer containing two proteins corresponding to a 10–15-fold excess over the capacity of the gelatin–Sepharose. The suspension was incubated for 16 h at 25 °C, and then centrifuged. Aliquots of the supernatant and gelatin–Sepharose were mixed with SDS/PAGE sample buffer. The composition of protein samples was analysed by SDS/PAGE using 11–22% (w/v) linear polyacrylamide gradient slab gels [27]. The gels were stained with Coomassie Brilliant Blue G-250 and densitometry of the stained gels was performed using a Varian 634 spectrophotometer equipped with a Varian gel scanner as described previously [25]. The ratios $[LP_i]/[LP_1]$ and $[P_1]/[P_i]$ were determined, and $K_i/K_1$ was calculated.

## RESULTS

Different fragments of the fibronectin-related part of human gelatinase A have been expressed in *E. coli*. Plasmids expressing recombinant proteins were constructed by exploiting restriction sites located or introduced at the boundaries of type II repeats, thus all constructs contain intact type II domains (Figure 1). Studies on recombinant proteins corresponding to different fragments of the gelatin-binding region of human gelatinase A have shown that each of the three type II domains is capable of mediating binding to gelatin–Sepharose 4B, facilitating their isolation by affinity chromatography (Figure 2). Binding of the expressed proteins to gelatin–Sepharose was found to be gelatin-specific as no adsorption to unsubstituted Sepharose 4B was observed. The gelatin-specific nature of the interaction is also supported by the observation that bound proteins could be specifically eluted with buffer containing 0.25% (w/v) gelatin (results not shown).

To isolate functionally homogeneous recombinant proteins, a second round of affinity chromatography on gelatin–Sepharose 4B was performed. In this step, proteins bound to gelatin–Sepharose 4B were eluted with a 0–8 M urea gradient to separate possible folding variants (Figure 3). In the case of DELβgalcoll 1, DELβgalcoll 2 and DELβgalcoll 3, the recombinant proteins eluted in single symmetrical peaks, i.e. they were homogeneous with respect to gelatin affinity (e.g. Figures 3a and 3b). Proteins DELβgalcoll 123, DELβgalcoll 23, DELβgalcoll 13 and DELβgalcoll 12, on the other hand, showed some heterogeneity (e.g. Figures 3c and 3d). A plausible explanation for the heterogeneity is that in proteins containing multiple type II domains, the population of gelatin-binding molecules may contain components in which some of the type II domains are improperly folded. Differences in the gelatin affinity of such folding variants could explain their different sensitivities to elution with urea. In accordance with this explanation, we have found that the major components were resistant to limited elastase digestion, whereas the minor components eluted at lower urea concentrations were degraded to smaller components (results not shown). In all subsequent experiments, only the major components eluted at high urea concentrations were used.

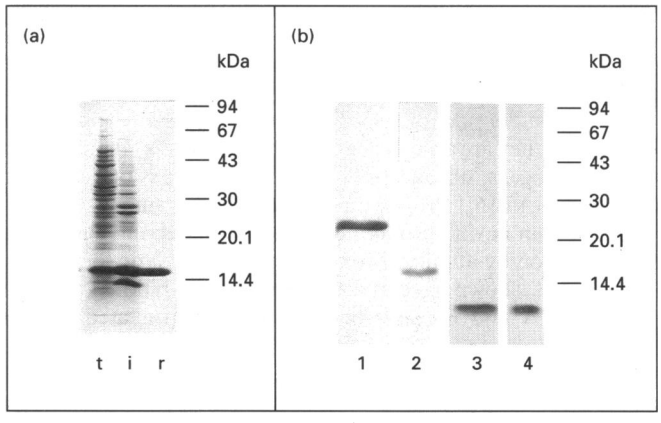


**Figure 2 Type II domains of human gelatinase A expressed in *E. coli***

(**a**) SDS/PAGE patterns of total bacterial proteins of isopropyl thiogalactoside-induced bacteria expressing DELβgalcoll 23 (t), proteins of isolated inclusion body (i) and the recombinant protein isolated by affinity chromatography on gelatin–Sepharose 4B (r). (**b**) SDS/PAGE analysis of recombinant proteins DELβgalcoll 123 (1), DELβgalcoll 12 (2), DELβgalcoll 1 (3) and DELβgalcoll 2 (4) isolated by gelatin–Sepharose affinity chromatography.

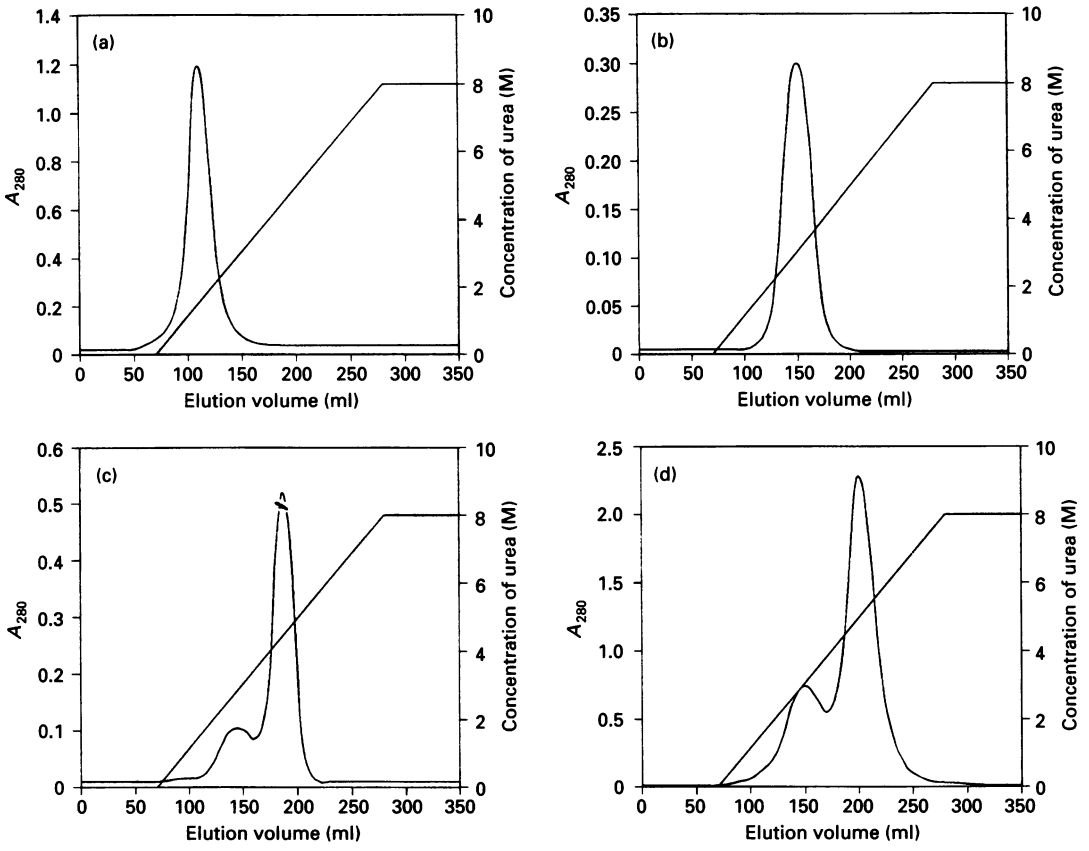


**Figure 3 Gelatin–Sepharose 4B chromatography of recombinant proteins containing type II domains of human gelatinase A**

Recombinant proteins DEL$\beta$galcoll 1 (**a**), DEL$\beta$galcoll 2 (**b**), DEL$\beta$galcoll 23 (**c**) and DEL$\beta$galcoll 13 (**d**) were applied onto gelatin–Sepharose 4B columns (3 cm × 10 cm) equilibrated with 100 mM Tris/HCl, 10 mM EDTA, pH 8.0, and chromatographed using an LKB h.p.l.c. system. The column was washed with one bed volume of the same buffer, and bound proteins were then eluted with a 0–8 M urea gradient, prepared by mixing 1.5 bed volumes of starting buffer with 1.5 bed volumes of buffer containing 8 M urea.

Studies on the interaction of recombinant proteins with gelatin–Sepharose revealed that binding is saturable (Figure 4). Scatchard plots of these data are curved (not shown), suggesting that gelatin–Sepharose possesses a range of binding sites with different affinities for the recombinant proteins. Comparison of the apparent association constants (Table 1) determined from half-maximal saturation points of the titration curves indicate marked differences in the binding of recombinant proteins to gelatin–Sepharose. Proteins DEL$\beta$galcoll 12, DEL$\beta$galcoll 23 and DEL$\beta$galcoll 13, containing two type II units, show significantly higher affinities for gelatin–Sepharose 4B than any of their constituent domains (DEL$\beta$galcoll 1, DEL$\beta$galcoll 2 and DEL$\beta$galcoll 3), and protein DEL$\beta$galcoll 123, which contains all three type II units, displays the highest affinity for gelatin (Table 1). These observations suggest that the three type II units of gelatinase A co-operate in gelatin-binding.

A similar conclusion could be drawn from the results of experiments in which overlapping fragments of gelatinase A competed for gelatin–Sepharose (Table 2). Protein DEL$\beta$galcoll 123, which contains all three type II units, competed most efficiently for gelatin: deletion of any one of the type II units (proteins DEL$\beta$galcoll 23, DEL$\beta$galcoll 13 and DEL$\beta$galcoll 12) decreased the affinity for gelatin (Table 2; rows 1–3). Similarly, fragments with two type II units (proteins DEL$\beta$galcoll 23 and DEL$\beta$galcoll 12) had higher affinity for gelatin than any of their constituent parts (Table 2; rows 4–8).

Surprisingly, pairs of non-overlapping fragments, such as DEL$\beta$galcoll 13/DEL$\beta$galcoll 2 or DEL$\beta$galcoll 23/DEL$\beta$galcoll 1, were also found to be able to displace each other from gelatin, suggesting that the different type II units may bind to the same sites on gelatin (Table 2; rows 9–10). These observations suggest that different type II units do not have unique binding sites: there is some promiscuity in their binding to gelatin.

It is worth highlighting the correlation between the affinity of recombinant proteins for gelatin–Sepharose and the urea concentrations at which the different recombinant proteins are eluted from gelatin–Sepharose 4B columns: the proteins that bind more tightly to gelatin–Sepharose are eluted at higher urea concentrations (Tables 1 and 2).

Experiments in which mixtures of the 42 kDa gelatin-binding fragment of human fibronectin and protein DEL$\beta$galcoll 123 were added to gelatin–Sepharose 4B revealed that the two proteins can displace each other from the affinant, raising the possibility that they recognize similar structures of gelatin. The gelatin-binding fragment of gelatinase A has approx. 9-fold higher affinity for gelatin than the 42 kDa fragment of fibronectin (Table 2; row 14).

## DISCUSSION

In this study, we have shown that each of the three type II domains contributes to gelatin binding of 72 kDa type IV collagenase (gelatinase A): they serve as subsites of an extended gelatin-binding site. It should be pointed out that our finding

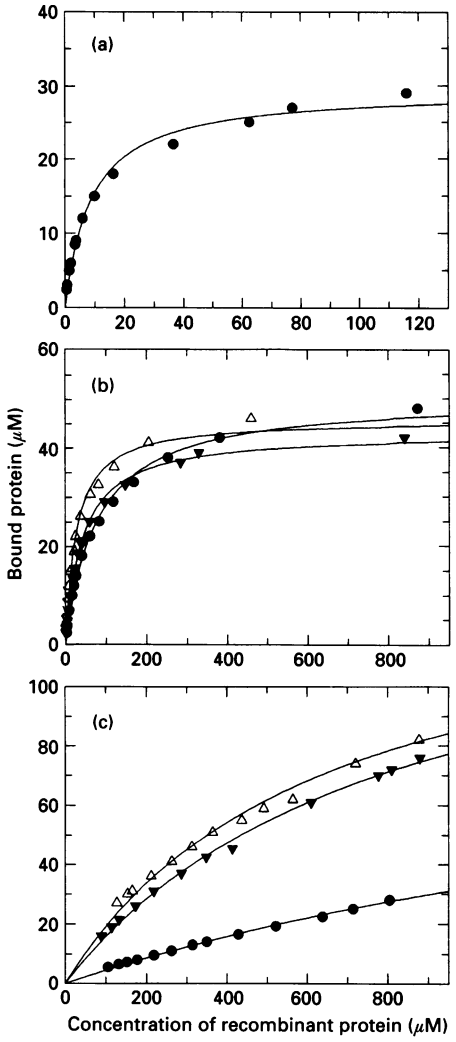


**Figure 4 Binding of recombinant proteins containing type II domains of human gelatinase A to gelatin–Sepharose 4B**

Different concentrations of recombinant proteins were mixed with 100 μl of gelatin–Sepharose 4B equilibrated with 100 mM Tris/HCl, 10 mM EDTA, pH 8.0, and the concentrations of the bound and free proteins were determined as described in the text. (**a**) DEL/βgalcoll 123 (●); (**b**) DEL/βgalcoll 12 (●), DEL/βgalcoll 23 (△), DEL/βgalcoll 13 (▼); (**c**) DEL/βgalcoll 1 (●), DEL/βgalcoll 2 (△), DEL/βgalcoll 3 (▼).

**Table 1 Affinity of type II domains of human gelatinase A for gelatin–Sepharose 4B**

| Recombinant protein | $10^3 \times K_a$ ($M^{-1}$) | Urea concentration at elution (M)* |
|---|---|---|
| DEL/βgalcoll 1 | 0.46† | 1.7 |
| | 0.35§ | |
| DEL/βgalcoll 2 | 1.58† | 2.9 |
| | 1.79‡ | |
| | 1.36§ | |
| DEL/βgalcoll 3 | 1.24† | 2.6 |
| | 1.36‡ | |
| DEL/βgalcoll 12 | 12.89† | 4.5 |
| | 17.38‡ | |
| DEL/βgalcoll 23 | 37.48† | 5.0 |
| | 35.88‡ | |
| DEL/βgalcoll 13 | 23.88† | 5.0 |
| DEL/βgalcoll 123 | 111.23† | 5.5 |

* Data refer to gelatin–Sepharose 4B chromatographies using urea gradients for elution of bound proteins (cf. Figure 3). The numbers indicate the urea concentration at the peak maxima of the eluted proteins.

† Apparent association constants determined from half-maximal saturation points of titration curves shown in Figure 4.

‡ Apparent association constant calculated from competition experiments using DEL/βgalcoll 123 as reference (cf. Table 2).

§ Apparent association constant calculated from competition experiments using DEL/βgalcoll 12 as reference (cf. Table 2).

**Table 2 Competition of fragments of human gelatinase A and fibronectin for gelatin**

The ratios of apparent association constants were determined as described in the Materials and methods section.

| | Competing species | | |
|---|---|---|---|
| Row | 1 | i | $K_i/K_1$ |
| 1 | DEL/βgalcoll 12 | DEL/βgalcoll 123 | 6.4 |
| 2 | DEL/βgalcoll 23 | DEL/βgalcoll 123 | 3.1 |
| 3 | DEL/βgalcoll 13 | DEL/βgalcoll 123 | 5.5 |
| 4 | DEL/βgalcoll 1 | DEL/βgalcoll 12 | 37.1 |
| 5 | DEL/βgalcoll 2 | DEL/βgalcoll 12 | 9.5 |
| 6 | DEL/βgalcoll 2 | DEL/βgalcoll 23 | 27.0 |
| 7 | DEL/βgalcoll 3 | DEL/βgalcoll 23 | 29.8 |
| 8 | DEL/βgalcoll 1 | DEL/βgalcoll 13 | 45.9 |
| 9 | DEL/βgalcoll 2 | DEL/βgalcoll 13 | 14.8 |
| 10 | DEL/βgalcoll 1 | DEL/βgalcoll 23 | 89.8 |
| 11 | DEL/βgalcoll 1 | DEL/βgalcoll 123 | ≫ 100 |
| 12 | DEL/βgalcoll 2 | DEL/βgalcoll 123 | 62.1 |
| 13 | DEL/βgalcoll 3 | DEL/βgalcoll 123 | 81.5 |
| 14 | 42 kDa fragment of fibronectin | DEL/βgalcoll 123 | 8.7 |

that the three type II units of the gelatinase A co-operate in gelatin binding differs from data obtained on gelatinase B [19]. In the latter work, it was found that the second type II domain binds most tightly to gelatin and the presence of the first and third type II domains actually weakens its gelatin affinity [19].

The finding that single type II domains of gelatinase A are able to mediate gelatin binding is in agreement with earlier observations that individual type II units of fibronectin, bovine seminal fluid protein PDC-109 and gelatinase B bind to gelatin [19,23]. N.m.r. studies on a type II unit of PDC-109 have identified several aromatic residues that participate in the construction of its gelatin-binding site [28,29]. In view of the fact that the same residues are also conserved in the other type II units, it seems possible that the gelatin-binding sites of fibronectin, PDC-109 and gelatinases are homologous. In view of our observation that the gelatin-binding fragments of fibronectin and gelatinase A compete for gelatin, we suggest that gelatinase A may bind to the same sites as fibronectin. It might be relevant in this respect that fibronectin binds to multiple sites of fibrillar collagens [30] and the structures of some of the high-affinity sites have been characterized [30–32].

The finding that non-overlapping fragments of gelatinase A (e.g. DEL/βgalcoll 13/DEL/βgalcoll 2 or DEL/βgalcoll 12/DEL/βgalcoll 3 in Table 2) displace each other from gelatin also suggests that the ligand specificities of different type II units may

be similar. The availability of recombinant type II domains will facilitate studies on their ligand specificities.

The function of the gelatin-binding site of gelatinases is unclear. It is probably not involved in determining the gelatin specificity of the enzymes because disruption of this interaction has no major effect on the gelatinolytic activity of gelatinase B [19]. A more likely possibility is that it targets gelatinases to sites where denatured collagen is formed/exposed as a result of the action of other proteases [33]. For example, proteases may produce fragments of collagen that spontaneously denature at body temperature and such denatured collagens could recruit progelatinases and gelatinases which, in turn, may degrade denatured interstitial collagens as well as collagen IV, collagen V, collagen VII, etc.

Our observation that the collagen-binding fragment of human gelatinase A has stronger overall affinity for denatured collagen than the gelatin-binding fragment of human fibronectin suggests that, even in the presence of fibronectin, gelatinase A may be efficiently targeted to sites where proteolytic degradation of the extracellular matrix has been initiated.

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