Binding of gelatinases A and B to type-I collagen and other matrix components

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Matrix sequestration of matrix metalloproteinases may be important for the facilitation of remodelling events and the migration of cells through the extracellular matrix. Using an ELISA technique we studied the ability of pro and active forms of gelatinases A and B (GLA and GLB) to bind to matrix components and the contribution made by the different enzyme domains. Pro and active forms of GLA and GLB bound to type-I and type-IV collagens, gelatin and laminin films. Binding to collagens occurred exclusively via the N-terminal portion of the molecule in both of the gelatinases; deletion of the fibronectin-like domain in GLA abolished binding. Fibronectin was shown to compete with GLA, confirming that binding occurs through this domain. GLA and GLB competed for binding to collagen type I, whereas collagenase and stromelysin bound to different sites and could be co-localized with the gelatinases. We conclude that gelatinases have different binding specificities from those previously documented for stromelysin and collagenase, which bind through their C-terminal domains to collagen fibrils.

INTRODUCTION

The matrix metalloproteinases (MMPs) comprise a gene family of enzymes that degrade components of extracellular matrix. The family includes two distinct interstitial collagenases of fibroblast/macrophage [1] and neutrophil origins [2], which catalyse the initial-rate-limiting step in the degradation of native collagens types I, II, III and X. Stromelysin, another member of this family, has broader substrate specificity that includes proteoglycans, laminin, fibronectin and types-IV and -IX collagens in their non-helical domains [3]. Two genetically distinct gelatinases, gelatinase A (GLA) and gelatinase B (GLB), have also been characterized which cleave native type-IV collagen molecules at a single site into one-quarter and three-quarter size fragments [4]. Both enzymes can degrade native types-V and -VII collagens, and have also been shown to have activity against denatured collagen (gelatin) and elastin [5–7]. GLA (72 kDa; MMP2) is secreted by cultured fibroblasts [8], cultured tumour cell lines [9,10], *ras*-transfected human bronchial epithelial cells [5], mesangial cells [11] and osteoblasts [12] and is synthesized during the early development of mouse embryos [13]. Expression of GLA correlates with metastatic ability in oncogene-transformed bronchial epithelial cells [14] and is linked with metastatic potential of many invasive human cancers [15,16]. GLB (92 kDa; MMP9) is secreted by neutrophils [17] and macrophages [18], and increased secretion of the enzyme has been reported in various malignant cells and *ras*-transformed murine cells [19,20] as well as in normal fibroblasts stimulated with phorbol 12-myristate 13-acetate, interleukin 1α or tumour necrosis factor [21]. GLA and GLB resemble each other with respect to primary structure and substrate specificity. The propeptide, gelatin-binding, Zn^{2+}-binding and C-terminal domains are highly homologous [5,22]. However, GLB contains a 54-residue proline-rich region which has no counterpart in GLA. This extra domain has sequence similarity to part of the helical domain of the type-V collagen α2 chain and many other collagens. Immunolocalization studies have shown that GLA, as well as collagenase and stromelysin, is able to bind to matrix components in rapidly resorbing tissues [23,24]. Previously we demonstrated that both collagenase and stromelysin bind to reconstituted type-I collagen fibrils [25–27]. By isolation of the different domains of the molecules, binding was shown to occur via the C-terminal domains. The C-terminal binding of collagenase is required for the specific cleavage of type-I collagen [27] whereas, although stromelysin also binds to collagen through this domain, cleavage does not occur. The physiological role of this matrix binding is still unclear, although the action of one enzyme on specific matrix components may be necessary to ensure the accessibility of other components to degradative enzymes. Localization of degradative enzymes in the matrix could concentrate the components of multistep enzyme pathways and thus facilitate their interactions.

Previous work by others focused on the gelatin-binding properties of the isolated gelatin-binding regions of GLA and GLB [28,29]. This study extends these observations by analysing the ability of latent and active forms of GLA and GLB to bind to various matrix components and the contribution made by the different enzyme domains. Enzymes lacking one or more domains were prepared using biochemical and protein engineering approaches. These included the C-terminal domain of GLA, GLA deletion mutants lacking either the C-terminal domain or the gelatin-binding region and GLB deletion mutants lacking either the C-terminal domain or both this domain and the collagen-like region. The results indicate that the binding characteristics for the gelatinases differ completely from those previously described for collagenase and stromelysin [26,27].

Abbreviations used: GLA, gelatinase (MMP2); GLB, gelatinase B (MMP9); MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

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MATERIALS AND METHODS

Preparation of enzyme forms

Recombinant wild-type human pro-GLA and des-(418–631)-GLA (comprising the catalytic and fibronectin-like domains) were purified from a myeloma cell expression system as described [8] and the C-terminal domain [des-(1–414)-GLA] was prepared biochemically [30]. Des-(418–631)-GLA has previously been shown to possess similar activities to the wild-type enzyme towards a number of substrates including type-IV collagen and gelatin [8]. The mutant form of pro-GLA from which the gelatin-binding region has been deleted, des-(191–364)-GLA, was prepared as described by Murphy et al. [31] and shown to have 50% of the activity of wild-type GLA against casein and 10% of the activity against gelatin. Recombinant wild-type human pro-GLB and des-(426–688)-GLB were prepared as described by O'Connell et al. [32]. Des-(471–688)-GLB was prepared in a similar fashion and was derived from the human pro-GLB cDNA by oligonucleotide-directed mutagenesis using PCR with the oligonucleotide 5′-ACCGGGGTGTCCAGGGGGGATCCGACC-GGGAAGATGCCGTGACTTAAGCGCGCG-3′ which introduced a stop codon and an *Eco*RI site after Pro-470. The specific activities of these GLB deletion mutants were similar to wild-type GLB using a quenched fluorescent peptide, gelatin or type-IV collagen as substrates [32] (G. Murphy, unpublished work). Recombinant human prostromelysin was expressed in C127 fibrosarcoma cells [33] and purified on Procion Red–Sepharose [34,35]. Recombinant human procollagenase was purified from NSO cell culture medium on S-Sepharose [27].

Activation of enzymes

GLA was activated by incubation with 2 mM 4-aminophenylmercuric acetate for 1.5 h at 25 °C. GLB was activated by incubation with active stromelysin at a molar ratio of 100:1 for 2.5 h at 37 °C.

Preparation of antibody recognizing the C-terminal domain of gelatinase

Three injections of recombinant human pro-GLA (each of 150 μg) were emulsified in complete Freund's adjuvant and injected subcutaneously and intramuscularly into multiple sites of a New Zealand White rabbit at fortnightly intervals. Two additional injections (each of 75 μg) of a mixture of both active and latent GLA were given emulsified in incomplete Freund's adjuvant and blood was taken 10 days after the last injections. IgGs were prepared by $(NH_4)_2SO_4$ precipitation and further purified by chromatography on DEAE-Sepharose. This antibody preparation fortuitously had undetectable levels of antibodies directed against the propeptide and N-terminal domain of GLA. Reactivities with wild-type GLA and des-(1–414)-GLA and lack of reactivity with des-(418–631)-GLA were confirmed by Western blotting.

Preparation of anti-peptide antibody (F14) to human GLB

Antiserum to human GLB was raised in rabbits by injecting the peptide RQRQSTLVLFPGDLRC (1 mg; residues 3 to 17 in the proenzyme plus a cysteine residue added at the C-terminus) conjugated to tuberculin (purified protein derivative) through the thiol group of cysteine using sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co.) and emulsified in incomplete Freund's adjuvant. Booster injections (0.5 mg) were given at 3-weekly intervals again using incomplete Freund's adjuvant, and blood was removed 10 days after each injection. Bleeds with the highest titres were determined by Western blotting and IgGs were prepared. Reactivity with GLB was determined by Western blotting, and immunolocalization was studied on NSO mouse myeloma cells transfected with GLB.

Immunoblotting

Samples were electrophoresed through polyacrylamide gels in the presence of SDS using the protocol of Laemmli and Favre [36]. Proteins were then electrotransferred to nitrocellulose and probed with either sheep anti-(human GLA) IgG [21] or rabbit anti-(human GLA) IgG which recognizes des-(1–414)-GLA. This was detected by peroxidase-coupled anti-sheep or anti-rabbit IgG respectively [37,38].

Evaluation of collagen binding using an ELISA technique

The polyclonal sheep antibodies to human GLA [21] and pig GLB [7] and the rabbit anti-peptide antibody F14, raised to the pro- piece of GLB, were used in three ELISAs to determine how well they recognized proenzymes relative to active forms, and the different enzyme domains. As no gelatinase antibodies were successful as 'capture' antibodies, the different gelatinase forms were coated directly on to microtitre plates. Samples were added to each well in 100 μl volumes of different dilutions and incubated overnight at 4 °C. Bound pro and active GLA and GLB and des-(418–631)-GLA, des-(1–414)-GLA, des-(191–364)-GLA, des-(471–688)-GLB and des-(426–688)-GLB were revealed using anti-(GLA) IgG, anti-(GLB) IgG (both at 20 μg/ml) or the anti-peptide antibody F14 (10 μg/ml) for 1 h at 25 °C. This was followed by either a donkey anti-sheep IgG (1:40000 dilution; Jackson Immuno-research Laboratories) or a goat anti-rabbit IgG (1:4000 dilution; Sigma Immunochemicals) conjugated to peroxidase for 1 h at 25 °C. 3,3′,5,5′-Tetramethylbenzidine (0.3 mM; Sigma) and 0.004% H_2O_2 (Sigma) in 0.1 M acetate buffer (pH 6.0) were used as substrates [39]. The reaction was stopped by the addition of 2.5 M H_2SO_4 and the absorbance at 450 nm was measured using an automated microplate reader model EL 310 (Bio-Tek Instruments). Comparison of the signal for pro and active forms of GLA using anti-(GLA) IgG showed that they were similar. Using the same antibody, the C-terminal domain of GLA [des-(1–414)-GLA] showed a slightly reduced signal compared with the N-terminal portion of the molecule [des-(418–631)-GLA], as did the gelatin-binding region deletion mutant [des-(191–364)-GLA] compared with wild-type pro-GLA. Pro and active forms of GLB showed a similar level of signal using anti-(GLB) IgG. Comparison of the signal for wild-type GLB, and the deletion mutants des-(471–688)-GLB and des-(426–688)-GLB, using the anti-peptide antibody F14 showed that the level of signal was similar in each case.

Collagen and gelatin film ELISAs

Type-I collagen films were prepared and their nativity (trypsin resistance) was determined as described [27]. The gelatin films were made in a similar way except that the type-I collagen was denatured by heating for 20 min at 60 °C. Gelatin was cross-linked to wells by adding 100 μl of 2% glutaraldehyde for 10 min at room temperature. Glutaraldehyde treatment of the gelatin film was shown to not affect its properties as a substrate. An equal volume of 0.1 M glycine was added to each well for a further 5 min to block unchanged groups. To prepare type-IV collagen films, type-IV collagen (Sigma) was dialysed into PBS

and diluted to 100 μg/ml before 50 μl was plated on to each well of a Nunc microtitre plate and dried at 37 °C. After the films had been washed (50 mM Tris/HCl, pH 7.6, 150 mM NaCl) to remove any crystals formed, pro and active forms of GLA and GLB, des-(418–631)-GLA, des-(1–414)-GLA, des-(191–364)-GLA, des-(471–688)-GLB and des-(426–688)-GLB were applied in 50 μl volumes in 100 mM Tris/HCl, pH 7.6, containing 150 mM NaCl and 0.2% casein to each well, and incubated for 1 h at 17 °C to prevent any degradation of the collagen or gelatin films. The films were then washed five times with 50 mM Tris/HCl, pH 7.6, containing 150 mM NaCl and 0.02% Tween 20 before bound enzyme was revealed using either anti-(GLA) IgG or anti-(GLB) IgG (both at 20 μg/ml), or the anti-peptide antibody F14 (10 μg/ml), for 2 h at 17 °C. This was followed by either donkey anti-sheep IgG (1:40000) dilution or a goat anti-rabbit IgG (1:4000) dilution. The substrates used were 3,3′,5,5′-tetramethylbenzidine and H_2O_2 as above.

Binding to other matrix components

Wells were coated with 50 μl of 100 μg/ml fibronectin, laminin or heparan sulphate (Sigma) in PBS. Proteoglycan monomer prepared from bovine nasal cartilage (a gift from Dawn Ward, Strangeways Research Laboratory) was also coated on to wells (0.5 mg/ml). Matrix components were allowed to dry at 37 °C before cross-linking. Non-specific sites were blocked by incubating with PBS containing 2% casein for 1 h at room temperature. Pro and active GLA and GLB were then applied in 50 μl volumes as described above. Bound enzyme was revealed using either anti-(GLA) IgG or anti-(GLB) IgG both at 20 μg/ml for 1 h at 17 °C, followed by donkey anti-sheep IgG conjugated to peroxidase (1:20000 dilution). The substrates used were 3,3′,5,5′-tetramethylbenzidine and H_2O_2 as above.

Competition experiments

For experiments using fibronectin as a competitor, 10 μg of fibronectin (Sigma) was preincubated with the collagen film (20 μg/ml) before the addition of pro-GLA. To determine the binding abilities of other MMPs to collagen films in the presence of gelatinases A or B, the collagen films were first saturated with pro-GLA, pro-GLB, des-(471–688)-GLB or des-(426–688)-GLB before incubation with prostromelysin, procollagenase or pro-GLA. Saturation was determined by ELISA and bound enzyme was revealed using anti-stromelysin IgG [26], anti-collagenase IgG [27] or anti-(GLA) IgG.

Tissue-inhibitor-of-metalloproteinases (TIMP)-binding experiments

To investigate the abilities of pro and active GLA–TIMP-2 and pro-GLB–TIMP-1 complexes to bind to collagen, gelatinase was incubated with a molar excess of each TIMP for 1 h at 25 °C to allow complexes to form before incubation with the collagen film. Recombinant human TIMP-1 was purified using a monoclonal antibody linked to S-Sepharose [40] and recombinant human TIMP-2 by the method of Willenbrock et al. [30].

RESULTS

Immunoblotting of isolated N- [des-(418–631)-GLA] and C-terminal [des-(1–414)-GLA] domains of GLA

Figure 1 demonstrates that the sheep anti-(GLA) IgG recognizes both N- and C-terminal domains of GLA, whereas the rabbit anti-(GLA) IgG (R123) is specific to the C-terminal domain.

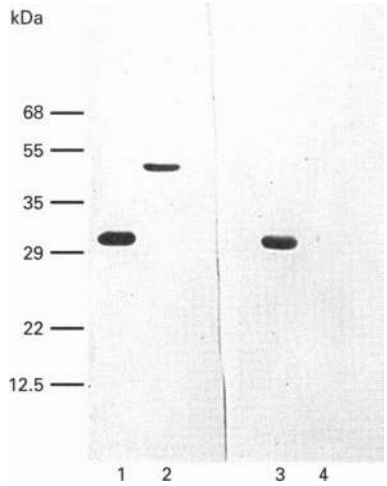

Figure 1 Detection on immunoblot of N- and C-terminal GLA by anti-(GLA) IgG

Samples of N-terminal GLA (lanes 2 and 4) and C-terminal GLA (lanes 1 and 3) were electrophoresed on a 10% polyacrylamide gel, immunoblotted on to nitrocellulose and probed with either sheep anti-(GLA) IgG (lanes 1 and 2) or rabbit anti-(C-terminal GLA) IgG (lanes 3 and 4) as described in the Materials and methods section.

GLA binding

We found that both pro and active forms of GLA bound to type-I and type-IV collagen, gelatin (denatured type-I collagen) and laminin films using an ELISA (Figures 2a–2d respectively). However, they bound very poorly to fibronectin and heparan sulphate and did not bind at all to the aggrecan prepared from bovine nasal cartilage (results not shown). More pro-GLA bound to type-I collagen (Figure 2a) and gelatin (Figure 2c) than the active form whereas the reverse was true for active GLA binding to type-IV collagen (Figure 2b) and laminin (Figure 2d). This discrepancy is probably explained by the conformations in which the enzymes bind to these different substrates and the reactivity of the antibody. To investigate further which particular domains of pro-GLA were responsible for binding to type-I collagen, isolated N- [des-(418–631)-GLA] and C- [des-(1–414)-GLA] terminal domains of pro-GLA were applied to type-I collagen films. Binding was found to occur via the N-terminal portion of the molecule (Figure 3). The inability of the C-terminal domain to bind was confirmed using the rabbit polyclonal antiserum which recognizes this domain (results not shown). Pro-GLA also bound to type-IV collagen via its N-terminus. Furthermore, deletion of the gelatin-binding region [des-(191–364)-GLA] abolished this binding (Figure 4).

When fibronectin (10 μg) was preincubated with the type-I collagen films as described in the Materials and methods section, pro-GLA binding was reduced at enzyme concentrations 0.3–100 nM (Figure 5). It could be calculated that when binding of pro-GLA to type-I collagen was 50% saturated, seven times more pro-GLA was required in wells preincubated with fibronectin to achieve the same level of binding as untreated wells.

Neither prostromelysin nor active collagenase was able to compete with pro-GLA for collagen binding. Preincubation of the collagen film with either prostromelysin or active collagenase had no effect on GLA binding, and *vice versa* (results not shown).

Complexes formed between pro and active GLA and TIMP-2

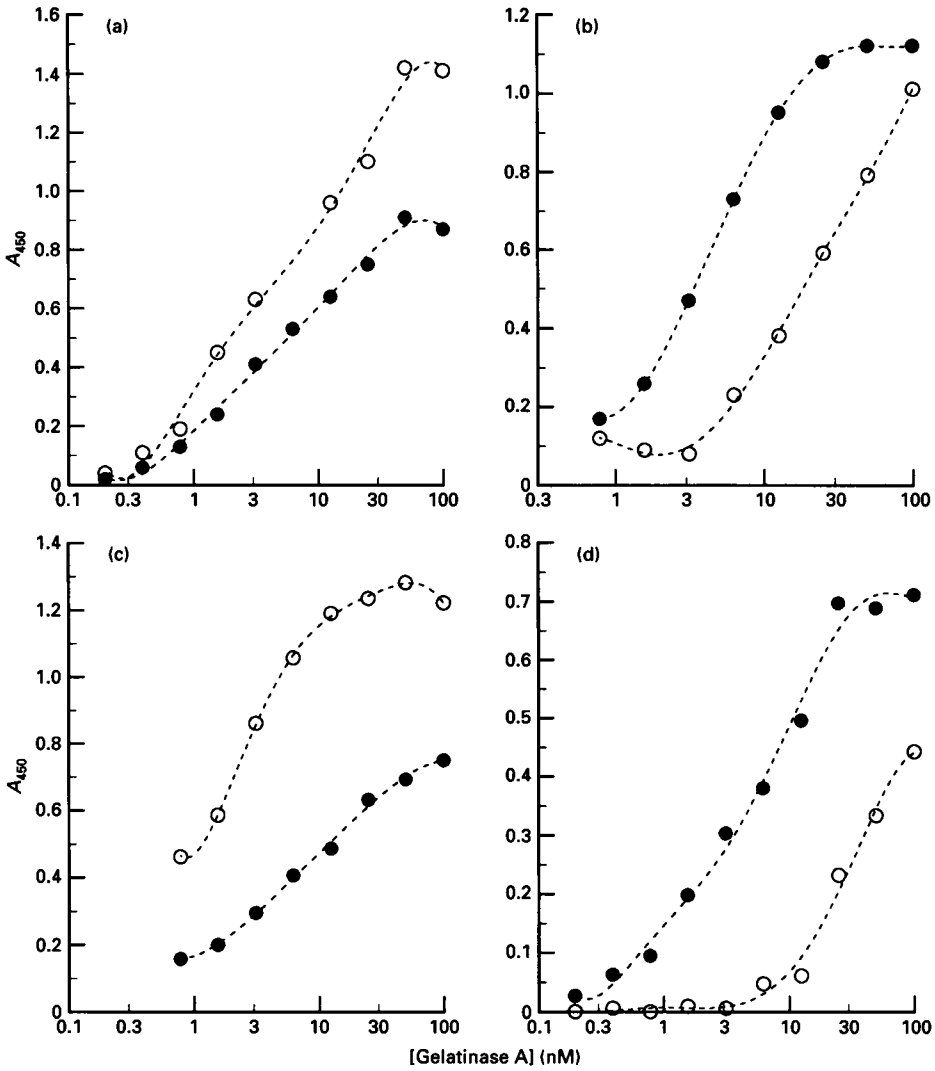

Figure 2 Binding of pro and active forms of GLA to matrix components

Pro (○) and active (●) GLA were allowed to bind to type-I collagen (**a**), type-IV collagen (**b**), gelatin (**c**) and laminin films (**d**) as described in the Materials and methods section before visualization with sheep anti-(GLA) IgG, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. Results are presented as A_{450} relative to the concentration of GLA incubated with the films. Each point is an average of duplicates from one of three essentially similar experiments.

failed to influence the binding of GLA to type-I collagen films (results not shown). The presence of complexes was confirmed using anti-TIMP-2 IgG [41].

GLB binding

Studies investigating GLB binding showed that, like GLA, both pro and active forms of the enzyme bound to type-I and type-IV collagens, gelatin and laminin (Figures 6a–6d respectively). Pro-GLB bound to type-I collagen (Figure 6a) and gelatin (Figure 6c) more avidly than the active form, whereas active pro-GLB bound to a greater extent than the pro form to type-IV collagen (Figure 6b). Using the anti-peptide antibody F14, which recognized all three forms of the enzyme equally well, the GLB deletion mutants, des-(471–688)-GLB and des-(426–688)-GLB, were shown to bind to type-I collagen, but less well than full-length enzyme (Figure 7). The mutant des-(426–688)-GLB displayed a lower level of binding than des-(471–688)-GLB. The decrease observed in binding of C-terminal-truncated GLB to type-I collagen at high enzyme concentrations was not observed with the ^{125}I-GLB mutants although the range of concentrations used was not so extensive (results not shown).

Pro-GLB did not compete with prostromelysin for collagen binding (results not shown). However, when the type-I collagen films were pretreated with pro-GLB this had the effect of substantially enhancing the binding of procollagenase. In contrast, when collagen films were pretreated with the GLB mutants, either des-(426–688)-GLB or des-(471–688)-GLB, the level of procollagenase binding was identical with that of the untreated collagen films (Figure 8a). When we investigated the ability of pro-GLA to bind to type-I collagen films pretreated with pro-GLB we found that pro-GLA binding was completely abolished (Figure 8b). Complexes formed between pro-GLB and TIMP-1 failed to influence binding of GLB to type-I collagen (results not shown) as was observed with GLA. The presence of pro-GLB–TIMP-1 complexes was confirmed using anti-TIMP-1 IgG

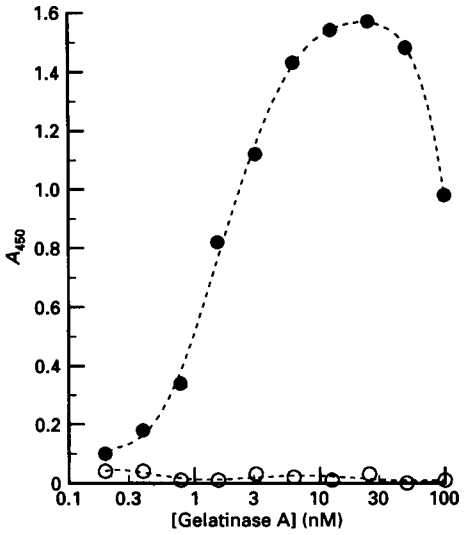

Figure 3 Binding of N- and C-terminal GLA to type-I collagen films

N-Terminal [des-(418–631)-GLA] (●) and C-terminal [des-(1–414)-GLA] (○) GLA were allowed to bind to type-I collagen films. Bound enzyme was revealed with sheep anti-(GLA) IgG, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. Results are presented as A_{450} relative to the concentration of enzyme incubated with the collagen film. Each point is an average of duplicates from one of three essentially similar experiments.

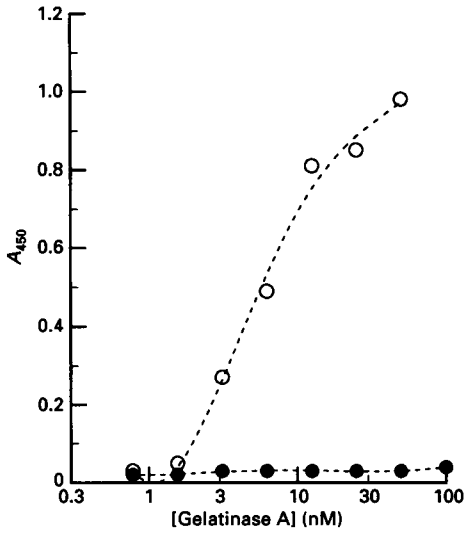

Figure 4 Binding of N-terminal [des-(418–631)-GLA] and the gelatin-binding region deletion mutant [des-(191–364)-GLA] to type-IV collagen films

N-Terminal [des-(418–631)-GLA] (○) and the gelatin-binding region deletion mutant [des-(191–364)-GLA] (●) were allowed to bind to type-IV collagen films. Bound enzyme was revealed with sheep anti-(GLA) IgG, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. Results are presented as A_{450} relative to the concentration of enzyme incubated with the collagen film. Each point is an average of duplicates from one of three essentially similar experiments.

[42] (results not shown). Binding of the progelatinase–TIMP-1 complex to collagen is not mediated by TIMP-1 since the latter molecule is unable to bind to type-I collagen [26].

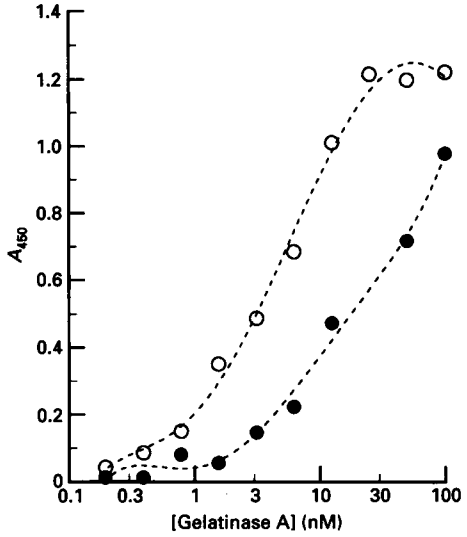

Figure 5 Competition between fibronectin and GLA for binding

Collagen films were incubated with (●) or without (○) fibronectin as described in the Materials and methods section before the addition of pro-GLA. Visualization of bound GLA was as described above.

DISCUSSION

In this study we have used previously characterized recombinant forms of GLA and GLB, in which specific domains have been deleted [8,31,32], to assess the contribution made by the different enzyme domains to binding to various matrix components. Using an ELISA technique we have shown that both pro and active forms of GLA and GLB bind to type-I and -IV collagens, gelatin and laminin. This is in agreement with immunolocalization studies [43] in which GLA was observed bound to matrix components in the synovium of patients with osteoarthritis and rheumatoid arthritis.

GLA was found to bind to type-I collagen exclusively via the N-terminal portion of the molecule. The inability of the C-terminal domain to bind was confirmed using the rabbit polyclonal antiserum R123 which recognizes this region. Also the observation that collagen binding was unaffected when pro-GLA was incubated with TIMP-2, an interaction mediated by the C-terminal domain of pro-GLA [44], further supports the conclusion that this domain is not involved in collagen binding. The GLB mutant, des-(471–688)-GLB, bound to collagen, demonstrating that the binding of this enzyme also occurs through the N-terminal region. In contrast with GLA and GLB we had previously found that stromelysin and collagenase bind to collagen via their C-terminal domains [26,27]; distinct sites on the collagen fibril are involved (J. A. Allan, A. J. P. Docherty, P. J. Barker, N. S. Huskisson, J. J. Reynolds and G. Murphy, unpublished work). This difference may be due to the different functions attributed to the C-terminal domains of these enzymes. Whereas the C-terminal domain of collagenase may play a role in substrate specificity [27,45,46], this region of the GLA molecule has been shown to be involved in activation of the enzyme on the membrane of fibroblasts stimulated with concanavalin A [8,47]. The inhibitors TIMP-1 and TIMP-2 have been shown to interact with the C-terminal domains of the latent forms of GLA and GLB respectively at a putative stabilization site [32,44,48,49] allowing enzyme–substrate interactions to be mediated by the N-terminal portion of the molecule.

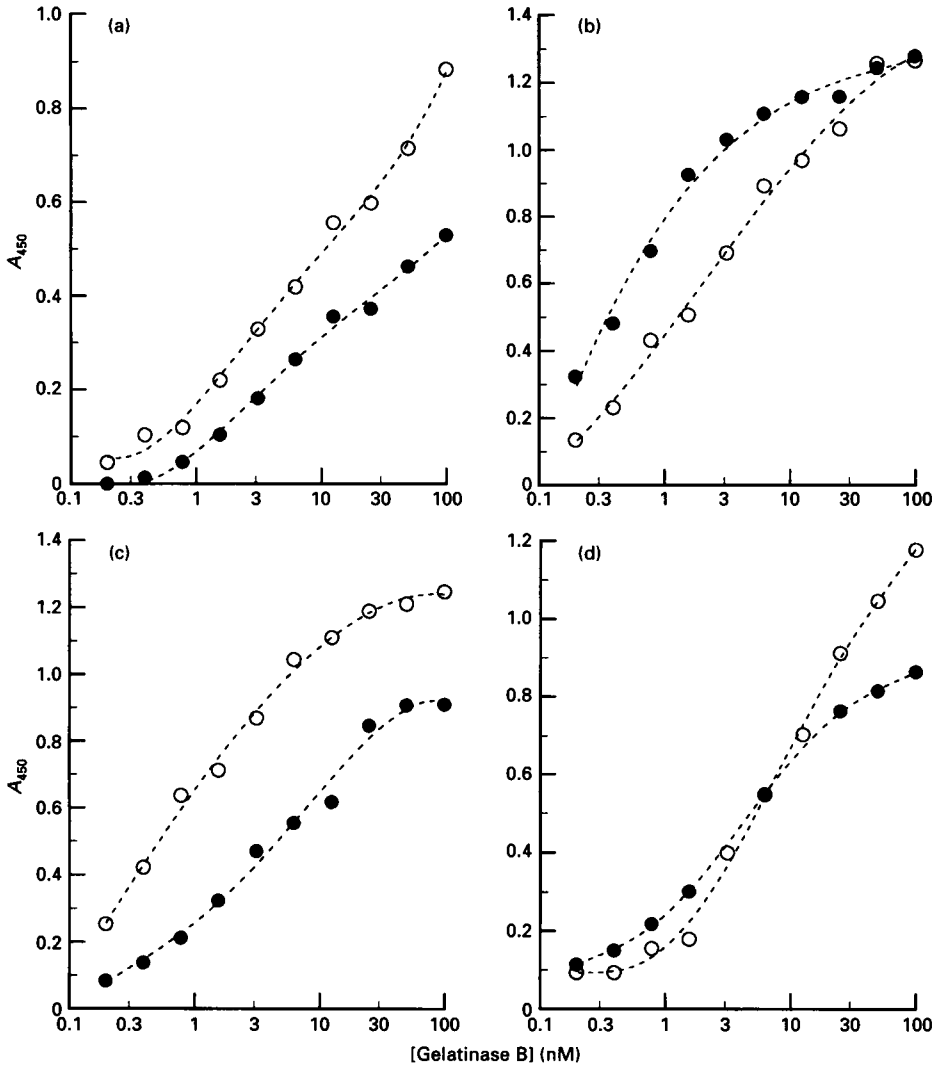

Figure 6 Binding of pro and active forms of GLB to matrix components

Pro (○) and active (●) GLB were allowed to bind to type-I collagen (**a**), type-IV collagen (**b**), gelatin (**c**) and laminin (**d**) films as described in the Materials and methods section before visualization with sheep anti-(GLB) IgG, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. Results are presented as A_{450} relative to the concentration of GLB incubated with the film. Each point is an average of duplicates from one of three essentially similar experiments.

The N-terminal portion of both GLA and GLB contain a domain which is absent from collagenase and stromelysin and consists of three repeats similar to those found in type-II domains of the gelatin-binding region of fibronectin [5,6]. Banyai and Patthy [28] have expressed the isolated fibronectin-like domain from GLA and shown that it binds to gelatin–Sepharose. We have shown that binding to type-I and type-IV collagens is abolished if this domain is deleted from GLA, indicating that the fibronectin-like domain is the sole site of collagen binding [31]. In addition, this domain has been demonstrated to be required for the specific cleavage of type-IV collagen [31]. In contrast, gelatinase binds to type-I collagen but this does not lead to cleavage. The fact that fibronectin preincubated with the collagen film is able to compete with GLA for binding suggests that this domain is responsible for collagen binding. Complete inhibition of gelatinase binding was not observed, which may be due to binding of GLA to fibronectin through other mechanisms or to weak interactions between type-I collagen and fibronectin that are competed for by GLA. As saturation of the collagen film with GLB also prevented GLA binding, we conclude that the two enzymes bind to the same site on the collagen fibril. This conclusion is consistent with evidence that the fibronectin-like domain common to both gelatinases is responsible for collagen binding .

Although Scatchard analysis of the binding of the gelatinases to collagens, gelatin and laminin was attempted, in this study it was not possible to determine K_d values for any of the interactions. This was attributable to both the low level of specific activity of the radiolabelled enzymes and the insufficient quantities of enzymes to allow studies exceeding the apparent K_d values for binding (10^{-7}–10^{-8} M). Furthermore, in previous work on the binding of collagenase to collagen fibrils [50], it was concluded that as little as 6% of the collagen may be so exposed as to be able to bind to enzyme. These studies were conducted with diffuse fibrils: it seems likely that our dried films of fibrils are even less accessible to enzymes, hence very few

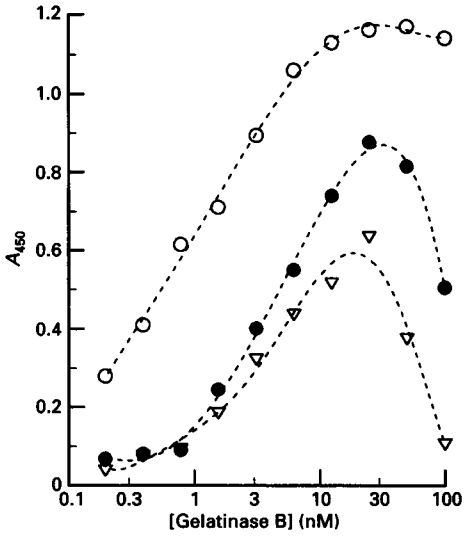

Figure 7 Binding of pro-GLB, des-(471–688)-GLB and des-(426–688)-GLB to type-I collagen films

Pro-GLB (○), des-(471–688)-GLB (● ○) and des-(426–688)-GLB (△) were allowed to bind to type-I collagen films. Bound enzyme was revealed with the peptide antibody, F14, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. Results are presented as A_{450} relative to the concentration of enzyme incubated with the film. Each point is an average of duplicates from one of three essentially similar experiments.

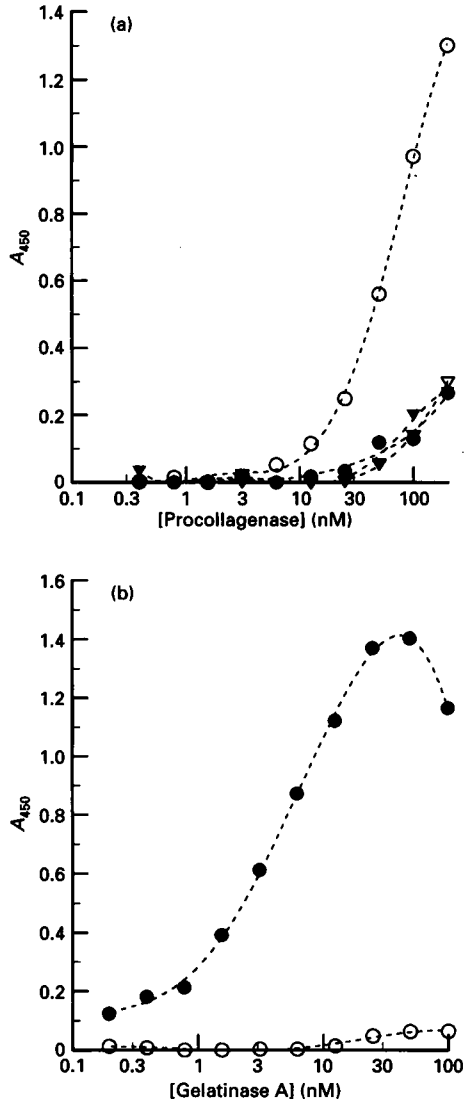

Figure 8 Binding of procollagenase or pro-GLA to type-I collagen films pretreated with pro-GLB or the GLB deletion mutants

(**a**) Collagen films were either untreated (▼) or pretreated with pro-GLB (○), des-(471–688)-GLB (●) or des-(426–688)-GLB (▽) before incubation with procollagenase. Bound procollagenase was revealed with sheep anti-collagenase IgG, peroxidase-labelled second antibody and 3,3′,5,5′-tetramethylbenzidine. (**b**) Collagen films were either untreated (●) or pretreated with pro-GLB (○) before incubation with pro-GLA. Bound pro-GLA was detected with sheep anti-(GLA).

potential binding sites are available. Collier et al. [51] and Strongin et al. [49] showed that the three contiguous copies of the fibronectin type-II homology unit bind to gelatin with different efficiences. A fusion protein containing the second homology unit bound severalfold more efficiently than that containing the third homology unit and the first homology unit fusion protein had negligible binding activity towards gelatin. In addition, the presence of the first and third type-II homology units actually weakened the gelatin affinity of the type-II unit. The 42 kDa gelatin-binding fragment of fibronectin which contains two type-II repeats homologous to those found in gelatinase has been shown to bind to collagen at two different sites with dissociation constants of 0.38 and 0.94 μM [52]. These data, however, differ from the recent work of Banyai et al. [29]. They have shown using GLA fragments that each of the three type-II domains has high affinity for gelatin, and fragments containing all three homology units had stronger affinity for gelatin than their constituent units indicating that they co-operate to form a high-affinity gelatin-binding site.

In contrast with GLA and B, which compete with each other for binding to collagen, collagenase and stromelysin are able to co-localize with either GLA or B on collagen. Co-localization on matrix of enzymes that act sequentially on a single substrate could increase the degradation of that substrate. Interestingly, we have shown that the binding of procollagenase to collagen is greatly enhanced when the collagen films are first saturated with GLB. Goldberg et al. [53] observed a stable pro-GLB–procollagenase complex under conditions of excess collagenase relative to GLB and TIMP-1. We, however, did not observe this increase in procollagenase binding when the collagen films were preincubated with the deletion mutants, des-(471–688)-GLB and des-(426–688)-GLB, indicating that procollagenase is interacting with the C-terminal region of pro-GLB. Goldberg et al. [53] also demonstrated that both components of this complex could be efficiently activated by plasmin and stromelysin. Both plasminogen and stromelysin have been shown to bind to extracellular matrix [26,27,54] and this concentration of proenzymes and activators on matrix could result in an increased rate of activation and reduced access of inhibitors. Furthermore, co-localization of enzymes on matrix could also increase degradation of substrate. This may be beneficial in the sequential action of collagenase and gelatinase on fibrillar collagens. On activation of collagenase, the native collagen can be cleaved to generate gelatin. Sequestrated gelatinase can then degrade this substrate to generate small collagen peptides.

In summary, we have demonstrated the ability of both GLA and GLB to bind to their substrates type-IV collagen, gelatin and laminin. In addition, the gelatinases bind to type-I collagen, even

though no cleavage occurs. Further research will be required to identify the location of these binding sites on collagen. Furthermore, we have shown that the enzymes interact with type-I and type IV-collagens via the N-terminal fibronectin-like domain and that a low-affinity binding site is involved in the interaction. The significance of gelatinase binding to collagenous matrix *in vivo* is unknown but we suggest that it may confer retention, stability and bioactivity for prolonged periods thus facilitating its role in pericellular proteolysis.

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