

The Hemopexin-like Domain (C Domain) of Human Gelatinase A (Matrix Metalloproteinase-2) Requires Ca^{2+} for Fibronectin and Heparin Binding

BINDING PROPERTIES OF RECOMBINANT GELATINASE A C DOMAIN TO EXTRACELLULAR MATRIX AND BASEMENT MEMBRANE COMPONENTS*

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U. Margaretha Wallon‡ and Christopher M. Overall§

From the Faculty of Dentistry and Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, 2199 Wesbrook Mall, Vancouver, British Columbia V6T 1Z3, Canada

The binding properties of the COOH-terminal hemopexin-like domain (C domain) of human gelatinase A (matrix metalloproteinase-2, 72-kDa gelatinase) were investigated to determine whether the C domain has binding affinity for extracellular matrix and basement membrane components. Recombinant C domain (rC domain) (Gly⁴¹⁷-Cys⁶³¹) was expressed in *Escherichia coli*, and the purified protein, identified using two antipeptide antibodies, was determined by electrospray mass spectrometry to have a mass of 25,925 Da, within 0.1 Da of that predicted. As assessed by microwell substrate binding assays and by column affinity chromatography, the matrix proteins laminin, denatured type I collagen, elastin, SPARC (secreted protein that is acidic and rich in cysteine), tenascin, and MatrigelTM were not bound by the rC domain. Unlike the hemopexin-like domains of collagenase and stromelysin, the rC domain also did not bind native type I collagen. Nor were native or denatured types II, IV, V, and X collagen, or the NC1 domain of type VII collagen bound. However, binding to heparin and fibronectin (K_d , 1.1×10^{-6} M) could be disrupted by 0.58–0.76 and 0.3 M NaCl, respectively. Using nonoverlapping chymotrypsin-generated fragments of fibronectin, binding sites for the rC domain were found on both the 40-kDa heparin binding and the 120-kDa cell binding fibronectin domains (K_d values, $\sim 4\text{--}6 \times 10^{-7}$ M). The Ca^{2+} ion, but not the potential structural Zn^{2+} ion, were found to be essential for maintaining the binding properties of the protein. The apo-form of the rC domain did not bind heparin, and both ethylenediaminetetraacetic acid and the specific Ca^{2+} ion chelator 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid, but not the Zn^{2+} ion chelator 1,10-phenanthroline, eluted the holo form of the rC domain from both heparin-Sepharose and fibronectin. Inductive coupled plasma mass spectrometry also did not detect a Zn^{2+} ion in the rC domain. In contrast, reduction with 65 mM dithiothreitol did not interfere with heparin binding, further emphasizing the crucial structural role played by the Ca^{2+} ion. Together, these data demonstrate for the first time that the he-

mopexin-like domain of gelatinase A has a binding site for fibronectin and heparin, and that Ca^{2+} ions are important in maintaining the structure and function of the domain.

The matrix metalloproteinases (MMPs)¹ constitute a family of proteolytic enzymes that together can degrade all components of the extracellular matrix and basement membranes, with each MMP having a distinct substrate preference (1, 2). MMP activity plays a major role during physiological and pathological processes, including embryogenesis, metastasis (3, 4), and inflammatory diseases (5, 6). Most soluble MMPs are secreted as proenzymes and share homologous primary and tertiary structures organized into distinct structural domains, with some differences in domain composition and number (6). These functionally and structurally defined domains include the NH₂-terminal zymogen domain containing the conserved PRGXP motif involved in enzyme latency (7) and a Zn^{2+} and Ca^{2+} ion binding catalytic domain. As with other proteinases, the specificity of peptide bond cleavage is determined by the S and S' subsite defining amino acid residues (8). Equally important are discrete substrate binding domains, or smaller functional modules, located outside of the active site, which form specialized exosites (9) to target proteolytic activity in tissues and are essential for cleavage of some substrates. Immediately adjacent to the catalytic site in gelatinase A (MMP-2, 72-kDa gelatinase) and gelatinase B (MMP-9, 92-kDa gelatinase) is a fibronectin type II-like module triple repeat (10, 11), which forms a collagen binding domain (CBD) with strong affinity for elastin and denatured types I (12, 13), IV, and V collagens, and native types I, V, and X collagens (9, 13),² proteins degraded by the gelatinases. Following the catalytic domain is a variably long linker, which in collagenase-1 (MMP-1) may be important for triple helix activity (14). The linker connects to the COOH-terminal domain (C domain), comprising four he-

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‡ Present address: Dept. of Biochemistry, University of Washington, Seattle, WA.

§ Recipient of a Medical Research Council of Canada Clinician Scientist Award. To whom correspondence should be addressed. Tel.: 604-822-2958; Fax: 604-822-8279; E-mail: overall@unix.ubc.ca.

¹ The abbreviations used are: MMP, matrix metalloproteinase; gelatinase A, also termed 72-kDa gelatinase, 72-kDa type IV collagenase, MMP-2; gelatinase B, also termed 92-kDa gelatinase, 92-kDa type IV collagenase, MMP-9; C domain, gelatinase A COOH-terminal domain; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; ConA, concanavalin A; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; rCBD, recombinant collagen binding domain of gelatinase A, consisting of fibronectin type II-like modules 1–3; rC domain, recombinant COOH-terminal hemopexin-like domain; SPARC, secreted protein that is acidic and rich in cysteine; TIMP, tissue inhibitor of matrix metalloproteinases; MT, membrane type.

² R. S. Abbey, B. Steffensen, and C. M. Overall, manuscript in preparation.

mopexin-like modules possessed by all MMPs except matrilysin (15). Three-dimensional structure analysis of the C domain of gelatinase A (16, 17) and collagenase-1 (18) has revealed a four-bladed β -propeller structure with a central Ca²⁺ ion, a potential Zn²⁺ ion binding site, and either a Ca²⁺-Cl⁻ (17) or a Na⁺-Cl⁻ (16) ion pair in the central channel.

Even though the primary and tertiary structures of the MMP C domains share extensive homology, they possess a range of different properties that are MMP-specific. The C domain of gelatinase B binds the natural tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) (11, 19, 20) whereas the C domain of gelatinase A is involved in binding to TIMP-2 (21) and to cell membranes (9, 22) on *concanavalin A* (ConA) activated normal cells (23) and tumor cells (24, 25). In addition, latent gelatinase A, in complex with TIMP-2, is activated on interaction with active membrane type MMPs (MT-MMPs) (26). Deletion mutants have demonstrated the requirement of the C domain of gelatinase A for cell binding and activation of the latent enzyme on cell surfaces (27), whereas the C domain of the collagenases mediates substrate specificity for native, triple helical type I collagen. In the absence of the C domain, the catalytic domain alone of collagenase-1 (28, 29) and neutrophil collagenase (30, 31) cleaves synthetic peptides and casein, but not native collagen. Indeed, the C domain alone of collagenase-1 binds native type I collagen (32–34), as can the C domain of stromelysin-1 (33, 34), even though collagen is not cleaved by stromelysin-1. In contrast, neither the activity nor the substrate specificity of the stromelysins is dramatically modified by the removal of the C domain (35–38). Likewise, the gelatinases degrade gelatin and native type IV collagen in a manner that appears independent of the presence of the C domain (39).

The gelatinases also efficiently degrade fibronectin, native types V, VII, and X collagen, and elastin (40–42). However, the importance of the C domain for these activities is unknown. Indeed, other than for elastin, native types I and V collagen, and gelatins that are bound by the CBD (12, 13, 42), exosites conferring specificity for these substrates have yet to be identified. The gelatinases may depend on the C domain for binding fibronectin or native type VII and X collagens as a requisite for efficient catalysis and triple helicase activity, but this has not been directly tested. In the present study we investigated the potential binding properties of a recombinant C domain of human gelatinase A for a number of extracellular matrix and basement membrane components. We found that the C domain exhibits strong binding properties for fibronectin and heparin and that the binding is dependent on the structural Ca²⁺ but not Zn²⁺ ion in the C domain.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Human types IV and V collagen (non-pepsin-treated) were from Becton Dickinson and Life Sciences; type X collagen and elastin were from Sigma; fibronectin fragments (40 and 120 kDa) and laminin were from Life Sciences; MatrigelTM was obtained from Becton Dickinson; tenascin was from Chemicon and Life Sciences; Affi-Gel heparin, Affi-Gel 10, and 10-DG columns were from Bio-Rad; chelating Sepharose 6B, gelatin-Sepharose 4B, heparin-Sepharose CL-6B, and CM-Sepharose Fast Flow were from Pharmacia Biotech Inc.; G-10 Sephadex, 1,2-bis(2-amino phenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), and keyhole limpet hemocyanin were from Sigma; and Freund's complete and incomplete adjuvants were from Calbiochem. Human gelatinase A cDNA was a kind gift from Dr. K. Tryggvason (Karolinska Institute, Stockholm, Sweden) (43) secreted protein that is acidic and rich in cysteine (SPARC) was kindly provided by Dr. J. Sodek (University of Toronto, Toronto, Ontario Canada); the NC1 domain of type VII collagen was generously provided by Dr. P. Rousselle (Centre National de la Recherche Scientifique, Lyon, France); and type II collagen was a gift from Dr. J. Mort (Shriners Hospital, Montreal, Quebec, Canada).

Acid-soluble native type I collagen was prepared from rat tail tendons by extraction with 0.5 M acetic acid and purified by differential

precipitation with 1.7 M NaCl (44). Fibronectin was affinity purified from bovine serum by binding to gelatin-Sepharose 4B and elution with a 0–10% Me₂SO gradient (45). The Me₂SO was then removed from the fibronectin using a 10-DG column. Purity was confirmed by SDS-PAGE analysis under reducing and nonreducing conditions.

Polyclonal Antipeptide Antibodies—Rabbit polyclonal α His₆ and α 72ex12 antipeptide antibodies were raised against a peptide present in the polyHis fusion tag and from part of a surface exposed β -strand of hemopexin module III of the gelatinase A C domain, respectively. Synthetic peptides were purified by high performance liquid chromatography before coupling to keyhole limpet hemocyanin (46). After emulsifying in Freund's complete adjuvant 1 mg of each peptide was injected subcutaneously into two rabbits each. Three to four biweekly boosts of peptide in incomplete Freund's adjuvant were administered intramuscularly. Whole serum was affinity-purified against peptide coupled to Affi-Gel 10 and quantitated by enzyme-linked immunosorbent assay.

Recombinant Gelatinase A Hemopexin-like C Domain Expression Construct—The 5'- and 3'-boundaries of the cDNA encoding the C domain and linker of gelatinase A (Gly⁴¹⁷-Cys⁶³¹) were defined by the 5'-boundary of exon 9 and the natural stop codon in exon 13, respectively. The cDNA was polymerase chain reaction-amplified from the full-length cDNA of human gelatinase A using the primers 5'-GCTAGCTAGCGGGGCCCTCTCTGACATT-3' and 5'-CTTAAGCTTCAGCAGCTAGCCAGTC-3', which added an *NheI* site and a *Bam*HI site in the 5'- and 3'-extensions, respectively. The purified 688-base pair product was digested with *NheI* and *Bam*HI and ligated into the expression vector pGYMX (13), which expresses recombinant protein with a short NH₂-terminal fusion tag comprising an initiation methionine, a His₆ tag, and a factor X_a cleavage site (13). Sequencing (47) of the plasmid, pGYMX9–13, confirmed the fidelity and reading frame of the cDNA.

Recombinant Protein Expression in *Escherichia coli*—*E. coli* strains were screened for recombinant protein expression after growth in 1% (w/v) tryptone (Becton Dickinson), 0.8% (w/v) yeast extract (Becton Dickinson), 0.5% (w/v) NaCl, and 100 μ g/ml ampicillin, pH 7.4. Log phase seed cultures were used to inoculate either 35- or 60-liter Chemap fermentor cultures, which were then grown under controlled constant temperature and aeration conditions for 24 h. Collected cells were washed and then lysed, inclusion bodies were dissolved in guanidinium-HCl, and recombinant protein was refolded prior to purification using procedures slightly modified (48) from those previously demonstrated (13) to produce correctly folded, disulfide cross-linked monomeric recombinant protein. In brief, disulfide bond exchange was performed in 0.1 M sodium borate, pH 10, under highly aerated conditions for 2 h, and the guanidinium was then slowly removed by step dialysis to refold the protein.

Purification of the Recombinant C Domain—Refolded protein was loaded on a Zn²⁺-charged chelating Sepharose 6B Column (V_r, 20 ml). After extensive washes with chromatography buffer (100 mM sodium dibasic phosphate buffer, 0.5 M NaCl, pH 8.0), nonspecifically bound bacterial proteins were eluted with a step pH gradient (pH 8.0–6.0) in 1.0 M NaCl. Recombinant protein was then eluted with a 0–400 mM imidazole gradient developed over 100 ml, and the pooled fractions buffer was exchanged to 50 mM Tris-HCl, pH 7.4, by gel filtration prior to snap freezing in liquid N₂.

SDS-PAGE and Enzymography—Heat-denatured protein samples were separated under reducing (65 mM DTT) or nonreducing conditions by SDS-PAGE according to the method of Laemmli (49) using 15% polyacrylamide gels. Protein bands were stained with Coomassie Brilliant Blue R-250. Samples analyzed by enzymography were electrophoresed nonreduced on 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin according to the method of Overall and Limeback (50).

Immunoblot Analysis—To confirm the identity of the purified protein, reduced and nonreduced recombinant protein was blotted onto a polyvinylidene difluoride membrane (Millipore), reacted with affinity-purified α His₆ or α 72ex12 antibodies, and then detected with peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence reagents (Amersham Corp.) on Kodak SB-5 film (Eastman Kodak Co.).

Mass Spectrometry—The mass of the rC domain was measured by electrospray mass spectrometry using a PESCIEX API 300 spectrometer after sample injection on a C18 high performance liquid chromatography column at 50 μ l/min. The Zn²⁺ ion content of the rC domain was measured three times by inductive coupled plasma mass spectrometry using 8.6–10 mg protein per analysis on a Sola (Finnigan-MAT) spectrometer.

Microwell Substrate Binding Assay—Binding of the rC domain to extracellular matrix proteins and substrates of gelatinase A was deter-

mined using an enzyme-linked immunosorbent type assay (13). Proteins tested were soluble fibronectin, laminin, SPARC, tenascin, native and denatured types I, II, IV, V, and X collagens, the NC1 domain of type VII collagen, and reconstituted basement membrane MatrigelTM. Denatured collagens were prepared by heat denaturation at 56 °C for 30 min. Ovalbumin was used as a negative control. To map C domain binding sites on fibronectin, nonoverlapping 40- and 120-kDa chymotrypsin fragments of fibronectin were used. Proteins (0.5 µg) in 15 mM Na₂CO₃, 35 mM NaHCO₃, and 0.02% (w/v) NaN₃, pH 9.6, were coated on 96-well microtiter plates for 18 h at 4 °C. Consistent and equal coating of test substrates in this assay has been previously confirmed (13). Wells were then blocked with 2.5% (w/v) ovalbumin, and serially diluted rC domain was added (1024 to 4 pmol/100 µl (10.24 µM to 40 nM) in 20 mM Tris-HCl, pH 7.4, for 1 h. Extensive washes with phosphate-buffered saline, 0.02% Tween 20 (v/v) followed, and the bound rC domain was then quantitated using either the αHis₆ or the α72ex12 antibodies followed by incubation with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody and *p*-nitrophenyl phosphate disodium as substrate. Assays were performed at least in duplicate per plate, and data were only compared for experiments on the same plate. All experiments were repeated six times, except for type II and X collagens, the type VII collagen NC1 domain, SPARC, and tenascin, which were performed in quadruplicate. Specificity was confirmed by comparing the binding of the rC domain with another recombinant domain from gelatinase A, the fibronectin type II-like triple repeat previously designated the collagen binding domain (13). The rCBD contains the same fusion tag and was expressed in the same *E. coli* strain used for rC domain expression.

Affinity Chromatography—Binding properties of the rC domain were also determined by affinity chromatography. Minicolumns containing either gelatin-Sepharose 4B, elastin-Sephadex G-10, heparin-Sepharose CL-6B, fibronectin coupled to gelatin-Sepharose 4B, or fibronectin coupled to Affi-Gel 10 (V_i, 100, 200, 100, 70, and 50 µl, respectively) were used in 50 mM Tris-HCl with or without 0.15 M NaCl, pH 7.4, chromatography buffer as appropriate (51). To ensure saturation of binding sites on the affinity matrix, a standardized quantity (50 µg, 2 nmol) of the rC domain was loaded onto the columns, which would result in excess protein being recovered in the unbound and wash fractions. To confirm binding to affinity matrices, any rC domain recovered in the unbound material was reapplied to another column of the affinity matrix, and binding was compared. Binding of the rC domain was typically assessed by step elution with NaCl to 1.0 M followed by a step gradient of Me₂SO (1–10%) in chromatography buffer. Chromatography fractions were analyzed by SDS-PAGE using 15% gels. Affinity chromatography experiments were performed at least six times for each matrix. FPLC was also performed using both heparin-Sepharose and Affi-Gel heparin 1.0-ml columns. After sample loading, a 0–1.0 M NaCl gradient was developed over 10 ml at 1.0 ml/min.

Chelation Experiments—To assess the role of the structural divalent cations on the binding of the rC domain to heparin and fibronectin, chelators (EDTA, 1,10-phenanthroline, EGTA, and BAPTA) were used to attempt elution from these affinity matrices. To confirm the elution by chelators, columns were then eluted with 1.0 M NaCl to recover any remaining bound rC domain. The apo rC domain was prepared before Affi-Gel heparin chromatography by chelation of structural Ca²⁺ ions for 1 h by 50 or 100 mM EDTA followed by desalting on a Sephadex G-10 spun column.

RESULTS

Characterization of the rC Domain of Human Gelatinase A—To investigate the properties of the hemopexin-like C domain of human gelatinase A, recombinant protein encoded by exons 9–13 of gelatinase A (Gly⁴¹⁷-Cys⁶³¹) was expressed in *E. coli*. The purified rC domain electrophoresed as a single band on 15% SDS-PAGE gels with an apparent molecular mass of ~26.5 kDa under reducing conditions (Fig. 1A). The absence of intermolecular disulfide linked multimeric forms of the recombinant protein was evident following electrophoresis under nonreducing conditions (Fig. 1A). The downshift in apparent molecular mass (Δ0.8 kDa) for nonreduced samples indicated the presence of an intact disulfide bond between Cys⁴⁴⁰ and Cys⁶³¹ (20) within the protein. The precise mass of the rC domain was measured to be 25,925.0 Da by electrospray mass spectrometry, within 0.1 Da of the predicted mass of a NH₂-terminal methionine-processed form of the recombinant pro-

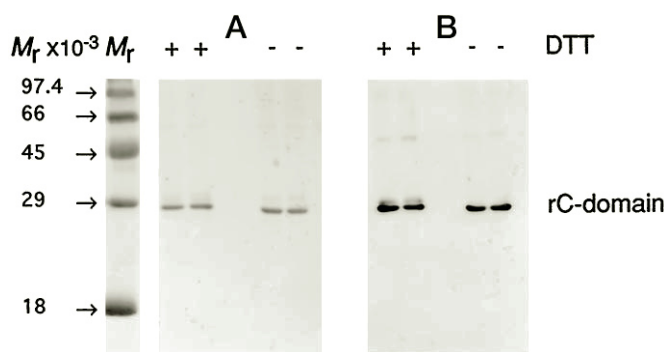


FIG. 1. SDS-PAGE and immunoblotting analysis of purified rC domain. Samples of the rC domain (2.5 µg/lane) were separated on 15% SDS-PAGE gels under reducing (+DTT) and nonreducing (−DTT) conditions in duplicate and then stained with Coomassie Brilliant Blue R-250 (A) or transferred to a polyvinylidene difluoride membrane by Western blotting (B). Immunoreactive protein was detected with affinity-purified α72ex12 peptide antibody as described under “Experimental Procedures.” *M_r*, reduced molecular mass marker proteins used here and elsewhere: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), horse heart myoglobin (18.4 kDa), chicken egg white lysozyme (14.2 kDa), and bovine insulin (6.2 kDa).

tein (25,924.9 Da), confirming the fidelity of correct expression. Immunoreactivity with two affinity purified antipeptide antibodies, α72ex12 (Fig. 1B) and αHis₆ (data not shown), further verified the identity of the purified protein. Importantly, essentially no disulfide cross-linked multimeric forms were observed on the immunoblots even when the ECL reaction was allowed to proceed well beyond the linear range.

Characterization of rC Domain Binding to Fibronectin, Laminin, SPARC, and Tenascin—Binding of the rC domain to basement membrane components was quantitated by the microtiter substrate binding assay. Although the rC domain did not bind MatrigelTM and laminin (Fig. 2A) or SPARC and tenascin (not shown), saturable binding to fibronectin in either NaCl-free 50 mM Tris buffer or phosphate-buffered saline was found, with an apparent *K_d* of 1.1×10^{-6} M. A recombinant collagen binding domain (13) from gelatinase A with the identical fusion tag did not bind fibronectin (Fig. 2B), further confirming the specificity of the rC domain interaction. Using nonoverlapping chymotrypsin-generated fragments of fibronectin, binding sites for the rC domain were found on both the 40-kDa heparin binding and the 120-kDa cell binding fibronectin fragments with a similar but slightly stronger (apparent *K_d* values, 4×10^{-7} and 6×10^{-7} M, respectively) affinity than for intact fibronectin (Fig. 3).

To further investigate the binding of the rC domain to fibronectin, the recombinant protein was applied to minicolumns of fibronectin coupled to gelatin-Sepharose columns in chromatography buffer containing 0.15 M NaCl. After adding the rC domain to saturation, as determined by the eventual detection of the recombinant protein in the unbound fraction (Fig. 4A, U), the bound rC domain was step eluted with 0.3–0.5 M NaCl in 50 mM Tris-HCl, pH 7.4. No further rC domain was recovered with 1.0 M NaCl or when Me₂SO was used to elute the gelatin-bound fibronectin (not shown). That the rC domain did not bind to the gelatin-Sepharose was confirmed in control experiments (Fig. 4B). Identical elution profiles were obtained when the rC domain was loaded in chromatography buffer with NaCl omitted (data not shown). To ensure that the small amount of unbound rC domain did not represent a misfolded form of the protein, this material was reapplied to another fibronectin affinity column. Fibronectin binding was again demonstrated (data not shown), confirming that the unbound rC domain was the result of overloading after saturation of fibronectin binding sites.

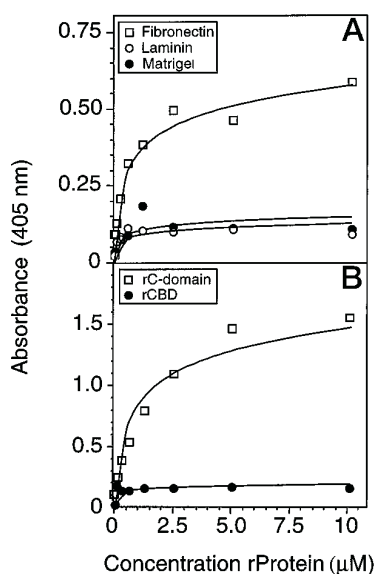


FIG. 2. Interaction of gelatinase A rC domain and rCBD with fibronectin, laminin, and Matrigel. Fibronectin, laminin, and MatrigelTM were coated on the bases of microtiter plate wells as described under "Experimental Procedures." Two-fold dilutions of the rC domain (10.24 to 0.04 μ M and 0 M) were added to the wells in a total volume of 100 μ l. To show the specificity of the rC domain interaction with fibronectin, the relative binding affinity of rCBD to fibronectin was also determined (B). The amounts of bound rC domain (A and B) and rCBD (B) were quantitated by reaction with affinity-purified α His₆-antipeptide antibody (which recognizes the same fusion tag on both the rC domain and rCBD) and alkaline phosphatase-conjugated secondary antibody. Data are plotted as the mean values of two experiments from the same plate. At saturation, rC domain binding to fibronectin compared with laminin and Matrigel (A) or fibronectin binding by rC domain compared with rCBD (B) was statistically highly significant ($p < 0.001$) as analyzed by the two-tailed Student's t test ($n = 12$).

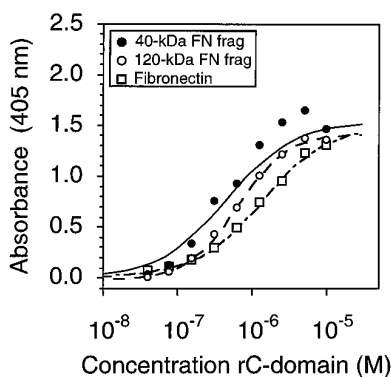


FIG. 3. Interaction of rC domain with fibronectin domains. Serially diluted rC domain was added to microwell plates coated with 0.5 μ g each of the 40- and 120-kDa fibronectin domains and intact soluble fibronectin as indicated. The bound rC domain was measured by enzyme-linked immunosorbent assay reaction with affinity-purified α His₆-antipeptide antibody. Curve-fitting and data points from the means of duplicate measurements from one enzyme-linked immunosorbent assay plate performed in triplicate are presented.

rC Domain Interaction with Type I, II, IV, V, VII, and X Collagens and Elastin—Although the C domains of collagenase-1 and stromelysin-1 bind native type I collagen, there was no binding of the gelatinase A rC domain to either native or denatured type I collagen in the microwell substrate binding assay compared with the negative control ovalbumin (Fig. 5A). The rC domain also did not bind gelatin-Sepharose (Fig. 4B), being quantitatively recovered in the unbound and wash fractions with no protein recovered in any elutes. In contrast, the rCBD from gelatinase A, used as a positive control for collagen

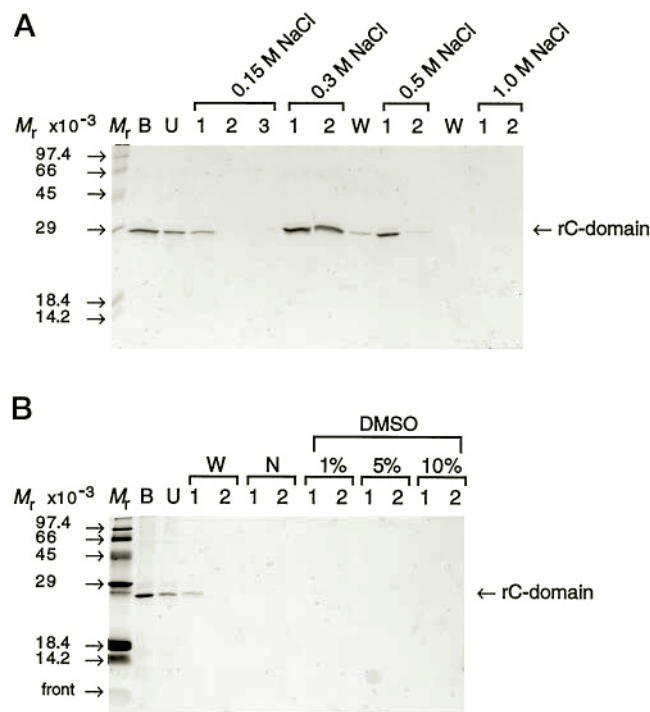


FIG. 4. SDS-PAGE analysis of fibronectin affinity chromatography of rC domain. Purified bovine fibronectin was coupled to gelatin-Sepharose 4B and packed into 70- μ l affinity chromatography mini-columns. To ensure saturation of binding to the fibronectin, the rC domain (B, before chromatography) was overlaid onto these columns (A) or gelatin-Sepharose control columns (B) until detected in the flow-through fraction (U, unbound). After extensive sequential washes with chromatography buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4), NaCl at 0.3, 0.5, and 1.0 M concentrations in 50 mM Tris-HCl buffer, pH 7.4, was then applied to the column with an intervening wash (W) in chromatography buffer as indicated. In B, columns were eluted first with 1.0 M NaCl (N) and then Me₂SO (DMSO) at the concentrations shown. Aliquots from each fraction were electrophoresed on 15% SDS-PAGE gels, and Coomassie Brilliant Blue R-250 was used for staining. M_r , molecular mass marker proteins in Da as indicated; front, dye front.

interaction, showed avid binding to both forms of type I collagen (Fig. 5A).

To determine whether the gelatinase A C domain contributes to the binding specificity for other substrates of the enzyme, the rC domain was tested for interaction with types IV and V collagen and elastin. As for type I collagen, the rC domain did not bind these collagens in either their native or denatured forms (Fig. 5B). Native and denatured types II and X collagen and the NC1 domain of type VII collagen were also not bound by the rC domain (not shown). In confirmation of these data using the α His₆ antibody, identical results were also obtained with the α 72ex12 antibody (not shown). rC domain binding to elastin was assessed using 6 mg of insoluble elastin mixed with 100 μ l of Sephadex G-10. All the loaded rC domain protein was recovered in the unbound and first wash fractions, and none was eluted with NaCl or Me₂SO (data not shown). This indicated that the rC domain does not contribute to the elastin binding properties of the enzyme that are localized to the fibronectin type II-like CBD of gelatinase A (13) and shown to be essential for elastinolysis by gelatinase B (42).

The rC Domain Binds to Heparin—Heparin-Sepharose mini-columns and FPLC columns were used to investigate the binding properties of the C domain of gelatinase A to the heparan sulfate component of basement membranes and cell surface proteoglycans. The bound rC domain was step eluted off heparin-Sepharose minicolumns with 0.5 M NaCl (Fig. 6A) and at 0.58 M NaCl on a NaCl gradient developed on a 1.0-ml heparin-Sepharose FPLC column (data not shown). Taking into consid-

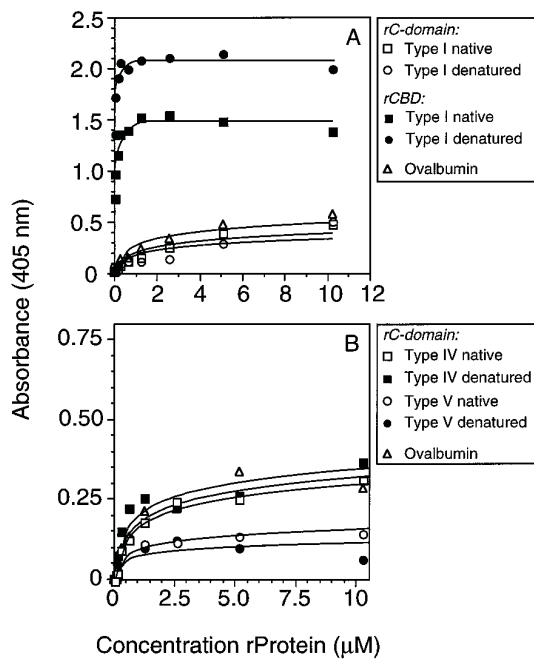


FIG. 5. Interaction of rC domain with native and denatured collagens. Microwell plate wells were coated with 0.5 μg of either native or heat-denatured type I collagen (A) or type IV or V collagens (B) with ovalbumin coated as a negative control. In A, after blocking and rinses, serial dilutions of rCBD (filled symbols) and the rC domain (open symbols) were added starting at 10.24 μM in a final volume of 100 μl. In B, binding of the rC domain to native (open symbols) and denatured (filled symbols) type IV and V collagens was assessed. Bound recombinant proteins were quantitated with affinity-purified αHis₆-antipeptide antibody as described under "Experimental Procedures." Data points are the averages of two experiments on the same plate. At saturation, rCBD binding to type I collagens compared with binding by the rC domain was statistically highly significant ($p < 0.001$) as analyzed by the two-tailed Student's t test ($n = 6$).

eration the calculated lag time for protein elution off the 1.0-ml column, the results of the minicolumn and FPLC were in accordance. Binding to Affi-Gel heparin was consistently stronger, requiring 0.76 M NaCl for peak elution (Fig. 7A). Heparin specificity was confirmed by the absence of binding to the negatively charged CM-Sepharose and that the unbound overloaded protein from the minicolumns could bind heparin-Sepharose on subsequent chromatography (data not shown).

To compare the heparin binding properties of the rC domain with gelatinase A, active enzyme was obtained from confluent cell cultures treated for 18 h with 20 μg/ml ConA (23) and then chromatographed over heparin-Sepharose minicolumns. Similar to the rC domain and confirming our previous studies (23, 51), the activated gelatinase A was also eluted off the column by 0.5 M NaCl (Fig. 6B). Previously we have shown that the gelatinase A CBD has a low affinity heparin binding site (13), and our recent mutagenesis studies³ have identified Lys³⁵⁷ as an essential residue in this site. Therefore, to compare the relative importance of this and other possible heparin binding sites on gelatinase A, active enzyme was loaded onto heparin-Sepharose, and competition was attempted with >20-fold molar excess rC domain added before elution was continued. Since only a small amount of the bound enzyme could be competed off the column by the rC domain, with the bulk of the enzyme being recovered in the 0.5 M NaCl elute (Fig. 6C), this indicated that other heparin binding sites on gelatinase A remained associated with the heparin. Since the previously described "mini-gelatinase," representing a ConA-induced processed form

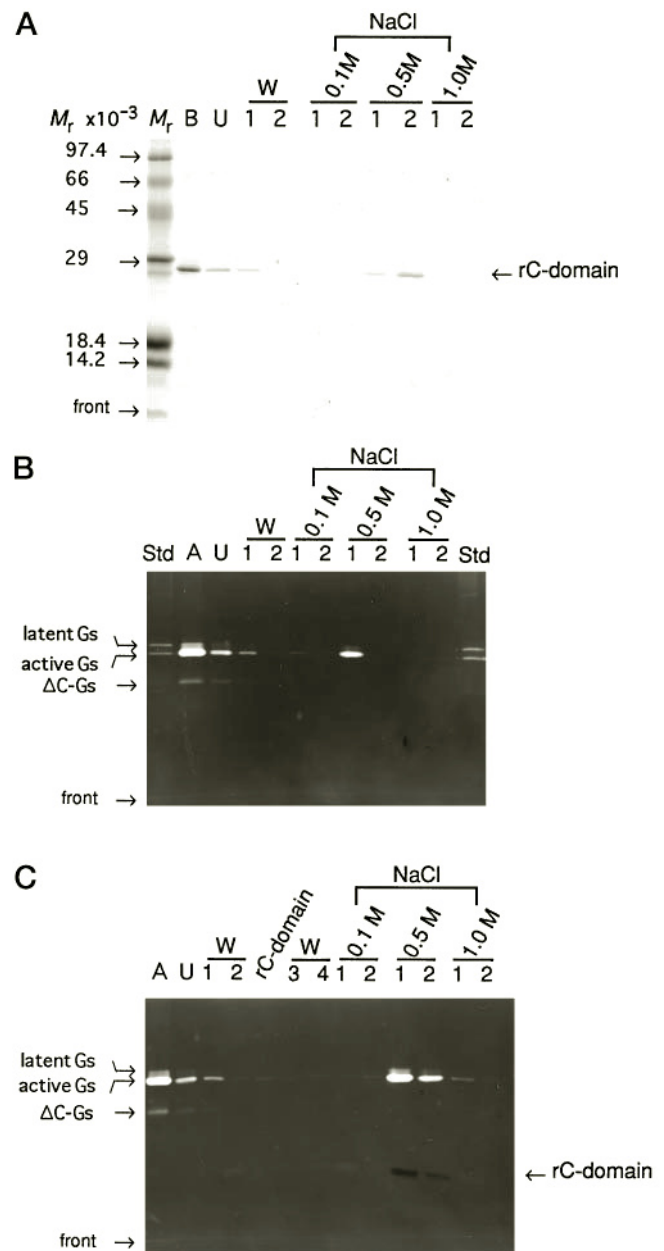


FIG. 6. Binding of rC domain to heparin. Minicolumns of heparin-Sepharose CL-6B ($n = 4$) were overloaded with 50 μg of the rC domain (A, lane B) and then washed (W) with chromatography buffer followed by elution of bound protein using a step gradient of NaCl as indicated. Aliquots from fractions 1 and 2 of each elute were analyzed by 15% SDS-PAGE. U, unbound protein; M_r , molecular mass marker proteins in Da as indicated; front, dye front. B, cell culture medium (500 μl) from ConA-treated ROS 17/2.8 cells (64) containing activated gelatinase A (lane A) was loaded onto heparin-Sepharose minicolumns ($n = 4$). After washes (W) with chromatography buffer, bound gelatinase A was eluted from the columns with a step gradient of 0.1–1.0 M NaCl, as before. Samples were separated on 10% SDS-polyacrylamide gels copolymerized with 1 mg/ml gelatin. After zymography, gelatinase activity was detected as cleared bands after staining with Coomassie Brilliant Blue R-250. Std, latent gelatinase A standard; ΔC-Gs, mini-gelatinase lacking the C domain produced by autolytic processing of the gelatinase A on ConA stimulation of cultures. In C, the rC domain was applied to a column of heparin-bound active gelatinase A ($n = 4$) in an attempt to compete for binding sites. After extensive washes (W, 3 and 4), bound proteins were eluted by a step gradient of NaCl as shown.

of gelatinase A lacking the C domain (23, 52) (Fig. 6, B and C, ΔC-Gs), was predominantly but not quantitatively recovered in the unbound and wash fractions, this reveals the importance of the C domain heparin binding site relative to the other heparin

³ B. Steffensen and C. M. Overall, manuscript in preparation.

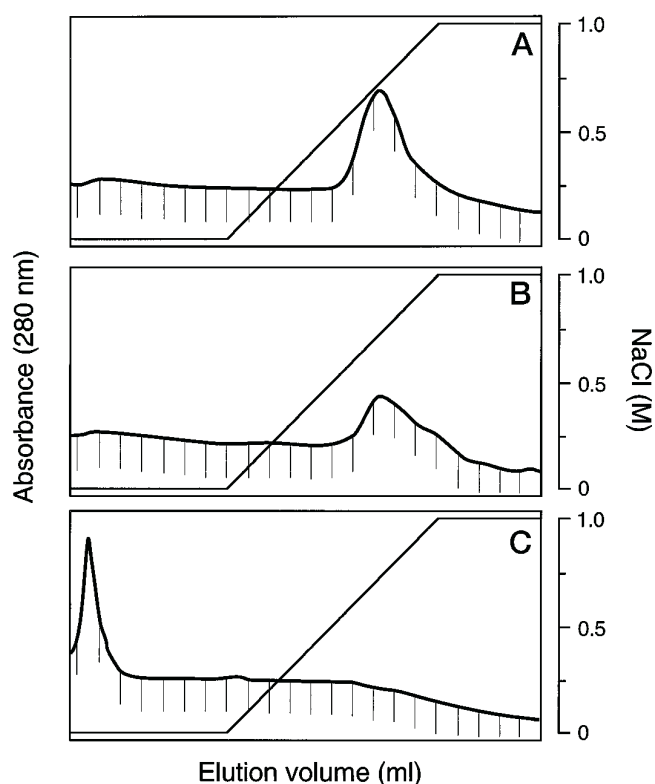


FIG. 7. FPLC elution profiles of reduced and apo-rC domain chromatographed on Affi-Gel heparin. The rC domain (A) was loaded at 0.5 ml/min on a 1.0-ml Affi-Gel heparin FPLC column equilibrated in chromatography buffer (50 mM Tris-HCl, pH 7.4) and eluted at 1.0 ml/min with a 1.0 M NaCl gradient. Protein elution was monitored by in line absorbance measurements at 280 nm with 1.0-ml fractions (hatch marks) collected after a 0.24-ml delay. Elution profiles of 100 μ g of the rC domain (A), the rC domain treated with 130 mM DTT for 1 h prior to chromatography (B), and the rC domain treated with 100 mM EDTA (C) are presented. The rC domain chromatographed in the presence of 0.15 M NaCl also bound Affi-Gel heparin and was eluted in an identical manner (not shown) as for the protein chromatographed in the absence of NaCl.

binding sites on human gelatinase A.

Structural Requirements for Heparin Binding—The structural importance of the Cys⁴⁴⁰-Cys⁶³¹ disulfide bond for heparin binding was examined after treatment of the rC domain with 65 or 130 mM DTT for 60 min prior to heparin-Sepharose chromatography. All minicolumn solutions also included freshly prepared 65 mM DTT to ensure that reducing conditions were maintained throughout chromatography. As shown in Fig. 8A, there was no change in the heparin-Sepharose elution profile of the reduced rC domain compared with unreduced samples (see Fig. 6A). When more precisely analyzed by Affi-Gel heparin FPLC, the reduced rC domain (130 mM DTT; Fig. 7B) was found to possess essentially identical elution properties as the unreduced protein (Fig. 7A) at 22 °C.

The recently published three-dimensional structures of the collagenase (18) and gelatinase A C domains (16, 17) show a divalent ion, modeled as a Ca²⁺ ion, in the central channel of the four-bladed β -propeller structure, together with either a Na⁺-Cl⁻ (16) or Ca²⁺-Cl⁻ (17) ion pair. In addition, a potential Zn²⁺ ion was also modeled in hemopexin module IV (16). In support of an important structural and/or functional role for the Ca²⁺ ions and a possible Zn²⁺ ion for binding of the rC domain to matrix components, the apo-rC domain was found to have lost binding potential for Affi-Gel heparin (Fig. 7C) and heparin-Sepharose (not shown) on FPLC. The loss of binding properties on removal of the structural Ca²⁺ was also confirmed by the elution of the heparin-Sepharose-bound holo-rC

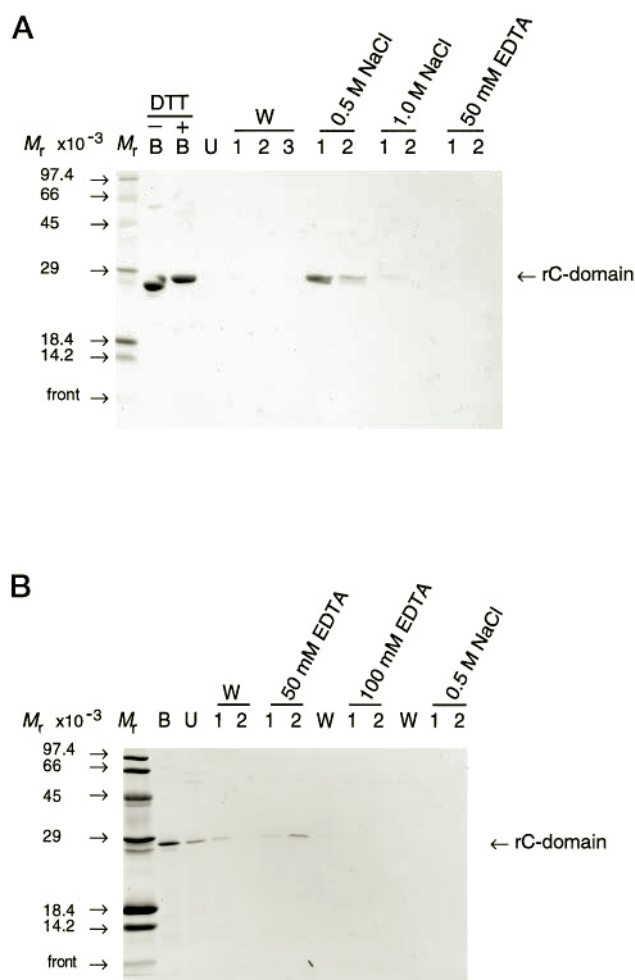


FIG. 8. Binding to heparin by rC domain is independent of disulfide bond integrity but divalent cation-dependent. The rC domain was reduced with 65 mM DTT for 1 h prior to chromatography (B, +DTT) over heparin-Sepharose CL-6B minicolumns (A). The column was overloaded until the rC domain appeared in the unbound fraction (U) followed by three 100- μ l washes (W) with chromatography buffer containing 65 mM DTT. Elution was as described in Fig. 6A with 0.5 M NaCl and 1.0 M NaCl as indicated, but with 65 mM DTT added to all solutions. The rC domain in column fractions migrated with an apparent molecular mass of 26.5 kDa compared with 25.7 kDa of non-reduced rC domain (B, -DTT) electrophoresed as a standard, confirming that the disulfide bond remained reduced during all chromatography steps. In B, excess rC domain (lane B; 50 μ g) was loaded onto heparin-Sepharose CL-6B minicolumns until the rC domain could be detected in the unbound (U) material. After washes (W), EDTA was applied to the columns with an intervening wash (W) in the absence of EDTA. To elute any remaining bound protein, 0.5 M NaCl in chromatography buffer was then added. Eluted protein was separated by 15% SDS-PAGE and then visualized by Coomassie Brilliant Blue R-250 staining. M_r , molecular mass marker proteins in Da as indicated; front, dye front.

domain by 50 mM EDTA on minicolumns (Fig. 8B). Elution was quantitative, with no further protein recovered by 100 mM EDTA or in the subsequent 0.5 M NaCl elution. Lower concentrations of EDTA (5, 10, and 20 mM) did not elute the bound rC domain (data not shown), suggesting a tight coordination of the divalent cations with the protein. Moreover, the rC domain treated with 15 mM EDTA prior to FPLC over Affi-Gel heparin retained heparin binding and elution properties identical to that of the holo protein (data not shown).

Identification of the Structural Divalent Cations—Since EDTA is a chelator of several divalent cations, including Ca²⁺ and Zn²⁺, more specific chelators were used to identify the important structural ions in the rC domain. The well charac-

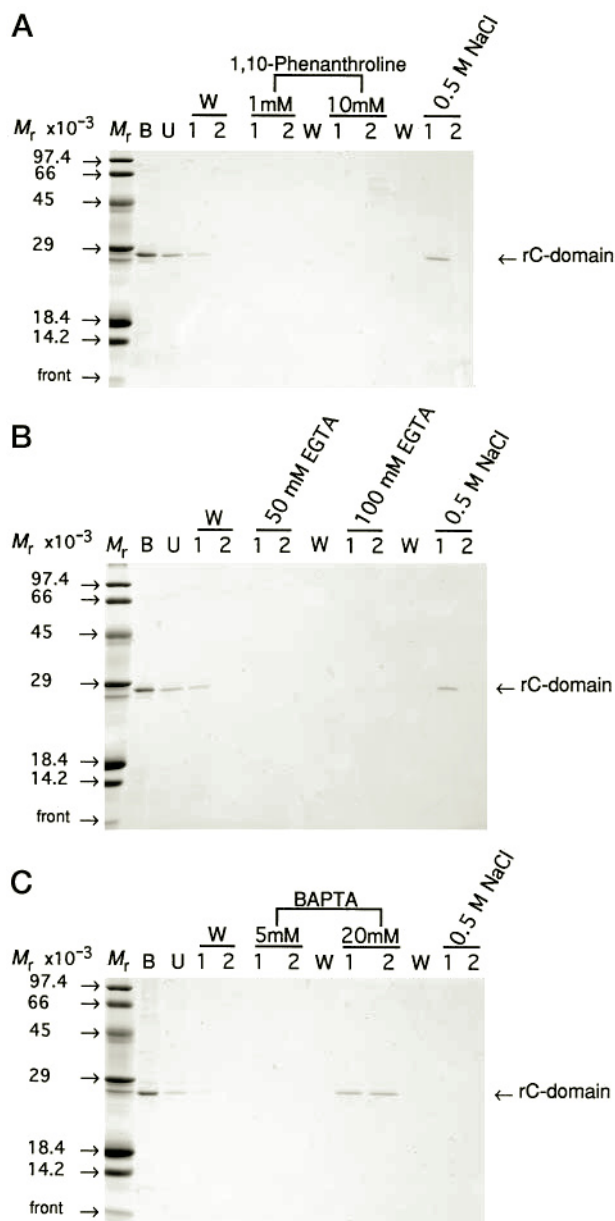


FIG. 9. Heparin binding by rC domain shows a Ca²⁺ but not a Zn²⁺ ion requirement. To investigate the role of a potential structural Zn²⁺ ion in the rC domain, the recombinant domain was bound to heparin-Sepharose, and after extensive washes (W) to ensure no carryover of overloaded unbound (U) protein, 1,10-phenanthroline (prepared from a stock solution of 1,10 phenanthroline dissolved in 70% (v/v) ethanol) was applied to the columns with an intervening wash (A, W). Finally, 0.5 M NaCl in chromatography buffer was applied to step elute any remaining bound rC domain (see Fig. 6A). *B* and *C* show the results using EGTA and BAPTA, respectively, for elution from heparin-Sepharose. Aliquots from each fraction, together with a sample of the rC domain before chromatography (*B*), were separated on 15% SDS-PAGE gels. *M_r*, molecular mass marker proteins in Da as indicated; *front*, dye front.

terized Zn²⁺ ion chelator 1,10-phenanthroline had no apparent effect on the binding of the rC domain to heparin, with all bound protein being recovered with the final 0.5 M NaCl elution (Fig. 9A). Thus, either no Zn²⁺ ions are normally ligated to the C domain, or if present, then chelation by 1,10 phenanthroline does not alter the binding properties of the protein. Moreover, inductive coupled plasma mass spectrometry analysis of the rC domain failed to detect a biologically significant Zn²⁺ ion content in the protein (8.5×10^{-6} mol of Zn²⁺/mol of rC domain).

Surprisingly, 100 mM EGTA was ineffective in eluting the rC

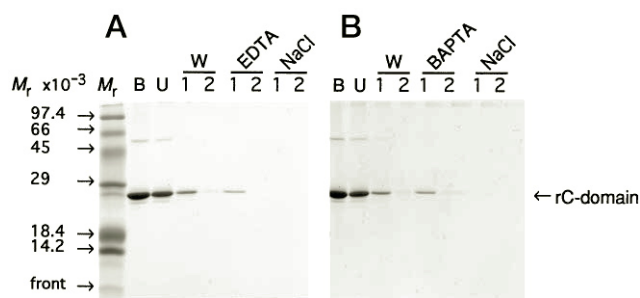


FIG. 10. Ca²⁺-dependent binding of rC domain to fibronectin. To determine whether the binding of the rC domain to fibronectin also requires structural Ca²⁺ ions, 50 μ g of the rC domain (*B*) was loaded onto mini-affinity columns of fibronectin coupled to gelatin-Sepharose 4B. The rC domain was overloaded until the protein appeared in the flow-through fraction (U) to ensure saturation of rC domain bound to fibronectin. After extensive washes (W), either 50 mM EDTA (A) or 20 mM BAPTA (B) was applied to the column. 1.0 M NaCl in 50 mM Tris-HCl buffer was then used to recover any residual bound protein. Aliquots were analyzed by 15% SDS-PAGE. *M_r*, molecular mass marker proteins in Da as indicated; *front*, dye front.

domain from heparin-Sepharose (Fig. 9B). This was confirmed by Affi-Gel heparin FPLC analysis in which elution was achieved only at 180–200 mM EGTA (not shown). Since EGTA is a Ca²⁺ and Mg²⁺ ion chelator, this result was paradoxical. However, several Ca²⁺ binding proteins are known to directly bind EGTA (53) due to the high electronegativity of the chemical. Therefore, to confirm the identity of the central Ca²⁺ ion, a newer and one of the most highly specific chelators of Ca²⁺ ions, BAPTA, was used. Low concentrations of BAPTA were ineffective in eluting rC domain (1 and 5 mM), but at 20 mM BAPTA (Fig. 9C) the bound rC domain was quantitatively eluted from the heparin-Sepharose. No further protein was recovered with 0.5 M NaCl (*cf.* 1,10-phenanthroline and EGTA; Fig. 9, A and B), confirming the importance and strength of association of the Ca²⁺ ion.

The rC Domain also Binds Fibronectin in a Ca²⁺ Ion-dependent Manner—To determine whether the structural Ca²⁺ ion has a similar influence on the binding properties of the C domain for fibronectin, minicolumns of fibronectin coupled to gelatin-Sepharose were loaded with the rC domain. Both 50 mM EDTA (Fig. 10A) and 20 mM BAPTA (Fig. 10B) quantitatively dissociated the rC domain from the fibronectin, with no further rC domain being recovered with 1.0 M NaCl. Overall, these experiments exclude the possibility of a structurally important Zn²⁺ ion in the rC domain, chemically identify the essential divalent cation in the rC domain as a Ca²⁺ ion, indicate the strength of the Ca²⁺ ion coordination with the protein, and reveal its important role in defining the binding properties of the rC domain to heparin and fibronectin.

DISCUSSION

Reported here are the fibronectin and heparin binding properties of the COOH-terminal hemopexin-like domain of human gelatinase A, the first such report for any of the MMPs. Fibronectin is a complex multidomain matrix glycoprotein with multiple binding sites for extracellular matrix components and cell membrane proteins. Indeed, the rC domain binds two non-overlapping chymotrypsin-generated fragments of fibronectin with similar *K_d* values (10^{-7} M range). The display of binding sites on fibronectin is conformation-dependent, with some cryptic sites being exposed on denaturation, surface binding, or cleavage (54), possibly explaining the slight difference in binding strength of the rC domain to the intact protein (apparent *K_d*, 1.1×10^{-6} M) compared with the 40- and 120-kDa fibronectin fragments. Fibronectin and its alternately spliced variants also occur as matrix-bound and soluble forms in serum. Accord-

ingly, the exact binding stoichiometry and dynamics of the gelatinase A C domain association with fibronectin in tissues is likely a complex interaction. Thus, although these experiments directly revealed the presence of multiple potential gelatinase A binding sites on the fibronectin chains, these sites may not always be available for concurrent occupancy due to structural differences between the intact tertiary fold of the protein and the cleavage fragments or to steric clashes, which may prevent multiple binding.

Fibronectin cleavage by gelatinase A results in prominent degradation fragments (40) that include the amino- and carboxyl-terminal fibrin binding domains and the larger central cell binding domain (54). Other fibronectin-degrading MMPs include the stromelysins, matrilysin, and metalloelastase (2), but as with gelatinase A, their fibronectin-degradative activities have received little study. With the exception of matrilysin, a fibronectin binding site might also be located on the C domain of these MMPs. Fibronectin binding may be a requisite for efficient catalysis, but previous C domain deletion mutant studies of gelatinase A (27, 55) or other MMPs (39, 55) did not investigate fibronectin binding or degradation. Fibronectin binding might also represent another means by which gelatinase A can bind cell surfaces. This may produce a reservoir of latent gelatinase A in proximity to the cell surface for eventual interaction with TIMP-2 and its receptor (56), poised for *cis* activation by MT-MMP-expressing cells (26, 57).

Gelatinase A also degrades native type IV basement membrane collagen and is considered pivotal for basement membrane remodeling during embryogenesis⁴ and in tumor cell invasion and metastasis (58). However, the rC domain did not bind basement membrane components either individually (type IV collagen, tenascin, laminin, and SPARC) or in combination as reconstituted basement membrane (MatrigelTM) or to the NC1 domain of anchoring fibril type VII collagen. This is consistent with the findings of others that C domain deletion mutants of gelatinase A did not bind (55) but still degraded (27) native type IV collagen and laminin. The rC domain also did not bind native type I, V, or X collagens, which are degraded by gelatinase A, or other collagens such as type II, which are not. Thus, the C domain of gelatinase A differs from those of the collagenases and stromelysins, which bind native type I collagen (32, 35–37, 55) and which, for the collagenases, are absolutely essential for collagenolytic activity (28–31). This indicates that the molecular mechanism involved in the triple helicase activity of gelatinase A for native type I, IV, V, and X collagens is fundamentally different from that of the collagenases. Indeed, a C domain deletion mutant of gelatinase A showed only slight alteration in type IV collagen cleavages (27). Alternatively, the collagen binding properties of the CBD of gelatinase A may compensate for the absence of collagen tethering by the C domain to potentiate triple helicase activity. Nonetheless, this does not explain the mechanism of type IV collagenolytic activity, since the isolated CBD does not bind native type IV collagen (13).

The heparin binding properties of gelatinase A have previously been described (51, 59) and shown to be important for enzyme activation (59). A gelatinase A heparin binding site has been previously located within the CBD (13).³ The heparin binding site located in the C domain is specific, since the rC domain did not bind and was only slightly retarded by interaction with the negatively charged CM-Sepharose. Although no match to the canonical heparin binding consensus sequences (XBBXB_X and XBBBXXB_X; B, basic amino acids; X, undefined)

(60) is found in the C domain, a site that matches the XBBXB_X site in reverse (SKNKKT) and a sequence with high similarity (VKKKMDPG) are located in the lysine- and arginine-rich hemopexin-like module III. However, competition experiments using an antipeptide antibody raised against this sequence (α 72ex12) had no effect on the binding of the rC domain to heparin (data not shown). These and other positively charged clusters within hemopexin-like modules III and IV are now being studied by site directed mutagenesis to identify the binding sites for heparin and the negatively charged COOH-terminal peptide of TIMP-2.⁵ Thus, heparan sulfate binding to the C domain may alter TIMP-2 interaction, which could in turn modulate cell surface activation of gelatinase A.

The Ca²⁺ ion dependence of the C domain interaction with fibronectin and heparan sulfate proteoglycans points to a new potential therapeutic target for gelatinase A. Confirmation of the importance and identity of the structural Ca²⁺ ions was shown in the apo form of the rC domain by disruption of the structural properties important for ligand binding. Chelation with EDTA and BAPTA, a new derivative of EGTA that is a highly specific Ca²⁺ chelator (61) but which has greatly improved rate constants and pH insensitivity over EGTA (61, 62), also eluted the bound holo-rC domain from heparin. The unexpected results obtained with EGTA may be due to direct association of EGTA with the protein (53) or the neutral pH used. At pH 7, EGTA occurs as a dianion, which results in a reduced ability to bind Ca²⁺ ions by 2 to 3 orders of magnitude compared with pH 8.6 (61). Together, these effects minimize the effective chelating capacity of the free EGTA for the heptacoordinated Ca²⁺ ion. Therefore, the chelation results obtained with BAPTA were important in clearly establishing the identity and importance of the Ca²⁺ ions in the rC domain. The chelation experiments further revealed the tight coordination of the Ca²⁺ ions in the protein, supporting the conclusion that the Ca²⁺ ions play an essential stabilizing role in the domain, possibly acting like a hub to centrally pin the four β -blades together. Last, since inductive coupled plasma mass spectrometry analysis did not measure any Zn²⁺ ions in the rC domain and 1,10-phenanthroline did not alter the binding properties of the rC domain, these data failed to confirm the presence of a structural Zn²⁺ ion in the C domain of gelatinase A. Together, these data indicate that the Zn²⁺ ion modeled in a potential binding site on module IV of the C domain was likely an artifact of crystallization in 150 mM Zn²⁺ acetate (16).

The central Ca²⁺ ion was also more effective than the disulfide bond in maintaining the structural integrity of the rC domain at 22 °C. Of note, these chromatography experiments were performed entirely under reduced conditions and not with reduced and alkylated protein. The introduction of blocking groups or a charged moiety during alkylation after reduction and protein denaturation by 6 M guanadinium or by heat can alter the structural properties of the protein distinct from those due to disulfide bond reduction alone. Indeed, reduced and alkylated rCBD from gelatinase A does not bind gelatin (13, 63), whereas rCBD protein chromatographed and analyzed under continuous reducing but nondenaturing conditions retains gelatin interaction equal to nonreduced rCBD (13). Thus, these experiments indicate that the integrity of the four-bladed β -propeller structure of the rC domain was not markedly perturbed at 22 °C by reduction of the disulfide bond but was altered sufficiently by chelation and loss of the structural Ca²⁺ ions to disrupt the heparin and fibronectin binding sites.

The widespread and near constitutive expression of gelatin-

⁴ A. Iamaroon, U. M. Wallon, C. M. Overall, and V. M. Diewert, resubmitted for publication.

⁵ C. M. Overall, A. King, M. Wallon, T. Lau, A. Ong, and D. Sam, unpublished data.

ase A indicates that the most important level of regulation of gelatinase A activity may be cellular activation and inhibition by TIMPs rather than by regulated transcription (1, 23, 65). In this regard, we have previously proposed that latent gelatinase A may bind native type I collagen through the CBD (13) and so may remain localized in tissues, poised for proteolysis of gelatin on collagenase cleavage of the native collagen and *trans* activation by other MT-MMP-expressing cells. A similar reservoir of enzyme may also be important for elastinolysis, since binding to elastin also occurs through the CBD of gelatinases (13, 42).² The binding of rC domain to fibronectin and potentially to heparan sulfate proteoglycans reveals additional extracellular or pericellular matrix components that may serve as anchors to sequester gelatinase A in tissues and to the cell surface. This may render the enzyme readily accessible to MT-MMPs for activation and to substrates, such as fibronectin and proteoglycan core proteins, on activation for cleavage. Together with the potential for the CBD to bind pericellular collagen,³ this also provides a novel additional mechanism for cell surface interaction that may complement those involving $\alpha_v\beta_3$ -integrin, MT-MMPs, and TIMP-2 receptors.

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