The Cysteine-rich Domain of Snake Venom Metalloproteinases Is a Ligand for von Willebrand Factor A Domains

ROLE IN SUBSTRATE TARGETING

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Snake venom metalloproteinases (SVMPs) are members of the Reprolysin family of metalloproteinases to which the ADAM (a disintegrin and metalloproteinase) proteins also belong. The disintegrin-like/cysteine-rich domains of the ADAMs have been implicated in their function. In the case of the SVMPs, we hypothesized that these domains could function to target the metalloproteinases to key extracellular matrix proteins or cell surface proteins. Initially we detected interaction of collagen XIV, a fibril-associated collagen with interrupted triple helices containing von Willebrand factor A (VWA) domains, with the PIII SVMP catrocollastatin. Next we investigated whether other VWA domain-containing matrix proteins could support the binding of PIII SVMPs. Using surface plasmon resonance, the PIII SVMP jararhagin and a recombinant cysteine-rich domain from a PIII SVMP were demonstrated to bind to collagen XIV, collagen XII, and matrilins 1, 3, and 4. Jararhagin was shown to cleave these proteins predominantly at sites localized at or near the VWA domains suggesting that it is the VWA domains to which the PIII SVMPs are binding via their cysteine-rich domain. In light of the fact that these extracellular matrix proteins function to stabilize matrix, targeting the SVMPs to these proteins followed by their specific cleavage could promote the destabilization of extracellular matrix and cell-matrix interactions and in the case of capillaries could contribute to their disruption and hemorrhage. Although there is only limited structural homology shared by the cysteine-rich domains of the PIII SVMPs and the ADAMs our results suggest an analogous function for the cysteine-rich domains in certain members of the expanded ADAM family of proteins to target them to VWA domain-containing proteins.

One of the hallmarks of viperid envenoming is local hemorrhage caused by the snake venom metalloproteinases (SVMPs) (1, 2). SVMPs are members of the Reprolysin subfamily of the M12 family of metalloproteinases (3). Of the SVMPs, the PIII class is distinguished by being comprised of protease, proteinase, disintegrin-like, and cysteine-rich domains (4). The proteinase domain of all the SVMP hemorrhagic toxins is believed to function to degrade capillary basement membranes, endothelial cell surfaces, and stromal matrix ultimately causing extravasation of capillary contents into the surrounding stroma (5, 6). Interestingly the PIII class of SVMPs is typically much more potent in causing hemorrhage compared with the PI and PII classes that lack the cysteine-rich domain found in the PIII class (4) suggesting a role for this domain in the pathophysiology of the PIII hemorrhagic toxins. Indeed the disintegrin-like/cysteine-rich domains of certain hemorrhagic toxins have been shown to be potent inhibitors of collagen-induced platelet aggregation as a result of interaction of the cysteine-rich domain with the α2β1 integrin on platelets (7, 8). Proteolytic degradation of capillary basement membrane structures and inhibition of platelet aggregation have been considered to be the key factors underlying the hemorrhagic potency of PIII SVMP hemorrhagic toxins (5, 9). Similarly recent studies from our laboratory demonstrated the ability of natural disintegrin-like/cysteine-rich domains processed from PIII SVMPs, as well as a recombinant cysteine-rich domain based on the structure from the PIII SVMP atrolysin A protease from Crotalus atrox venom, to support the interaction of the PIII SVMPs with von Willebrand factor (10). Thus, these studies indicate that the non-protease domains, particularly the cysteine-rich domain of the PIII SVMPs play an important role in the pathophysiology of the toxins targeting the proteinase domain to rel...
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Cell Membrane Preparations—Human HS68 fibroblasts grown to 80% confluence in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum were detached from tissue culture plates by a rubber policeman, suspended in phosphate-buffered saline (PBS), and centrifuged. The cell pellet was resuspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and homogenized with a Polytron for 30 s at 0 °C. The homogenate evant substrates to promote hemorrhage. This leads to the question of whether there are conserved structural motifs in other relevant proteins that can support PIII SVMP binding and thus targeting proteolysis by the SVMPs.

von Willebrand factor (VWF), a plasma and extracellular matrix protein, contains three von Willebrand factor A domains (referred to as VWA1, VWA2, and VWA3). VWA domains are involved in cell adhesion and are present in extracellular matrix proteins and in integrin receptors (11). Among the VWA-containing proteins are the fibril-associated collagens with interrupted triple helices (FACITs) that form a subclass of collagens characterized by the presence of more than one triple helical domain separated by non-triple helical segments. FACIT collagens XII (12) and XIV (13, 14) have one domain that anchors the molecule to the surface of the fibril and three “finger-like” domains containing VWA-like regions (15).

Among the non-collagenous VWA-containing extracellular matrix proteins are the matriline. Matrilins 1, 2, and 4 contain two VWA domains that flank a variable number of EGF domains, whereas in matrilin 3 the C-terminal VWA domain is missing. Matrilins are thought to play a role in the formation of fibrillar or filamentous structures associated with collagens (16).

To further elucidate the structural features of PIII SVMPs and their ability to target physiological substrates as well as to gain new insights into the mechanisms of hemorrhage production by these toxins we initially undertook in vitro studies to explore the interacotme of Western diamondback rattlesnake (C. atrox) venom proteins with fibroblast cell surface- and cell surface-associated proteins. Based on our studies we now report that an extracellular matrix/cell surface-associated protein, collagen XIV, was identified as a binding partner of a PIII SVMP, catrochallastatin from C. atrox venom. The general ability of PIII SVMPs to interact with collagen XIV was confirmed using another PIII SVMP, jararhagin, isolated from Bothrops jararaca venom with surface plasmon resonance studies. This interaction phenomenon was extended to another FACIT collagen, XII, as well as the extracellular matrix proteins matrilin 1, 3, and 4, all proteins containing VWA domains. We also demonstrated that interaction of the PIII SVMPs with these proteins was mediated via the cysteine-rich domain that serves to target SVMP proteolysis to peptide bonds at or near their VWA domain-binding site in these proteins. In summary, we have identified novel binding partners/substrates for PIII SVMPs that use the cysteine-rich domain to mediate binding to substrate VWA domains and target them for proteolytic degradation. A role for collagen XII and XIV and matrilin 1, 3, and 4 degradation in SVMP-induced hemorrhage is unknown; however, given their presence in the collageneous stroma surrounding capillaries in many tissues and their role in matrix stabilization, one can speculate on their importance to capillary integrity, and thus their degradation by the hemorrhagic SVMPs may contribute to hemorrhage production via capillary destabilization.

Experimental Procedures

Proteins

Recombinant A/C (cysteine-rich domain protein from atropyalin A) was prepared as described elsewhere (10); jararhagin, a PIII hemorrhagic SVMP from B. jararaca venom (17), was a kind gift from Dr. Ana M. Moura-da-Silva (Instituto Butantan, São Paulo, Brazil). Bovine collagen XII and collagen XIV were prepared as described before (18). Collagen XII as isolated from bovine tissue represents the small splice variant. This variant lacks the first 1189 amino acid residues and starts with the third VWA domain. Native matrilin 1 was extracted from fetal bovine rib cartilage as described previously (19); recombinant full-length matrilins 2, 3, and 4 (19) and VWA matrilin domains 4A1 and 4A2 were prepared as described previously (19). The cDNA encoding the matrilin 3 VWA domain was a kind gift from Dr. Patric Nitsche (Lund University, Lund, Sweden) and was amplified by PCR using primers that inserted an SpeI restriction site at the 5′-end and a NotI site at the 3′-end, respectively (matn3A1m forward, 5′-GCCCACCTGTTCGAAAGCCAGGCCTTGTG-3′; and matn3A1m reverse, 5′-CAA-TGACTGCGGCGCTTAAAGCACAAGGGTTCTCGGATC-3′). After digestion with SpeI and NotI, the amplified cDNA fragment was inserted into the expression vector pCPE-Pu with a C-terminal His tag in-frame with the signal peptide of BM40 (20). The cDNA encoding the matrilin 4 EGF domains was amplified by PCR using primers that inserted an SpeI restriction site at the 5′-end and a BamHI site at the 3′-end, respectively (matn4EFGm forward, 5′-GCCCACTAGTAAAGGACCTGTGTGCTGAATGG-3′; and matn4EFGm reverse, 5′-CAATTGGATCCCGGGTCACAAGCTCTGGCCATC-3′) and inserted into the expression vector pCPE-Pu with a C-terminal His tag in-frame with the signal peptide of BM40 (20). cDNA coding for the fibronectin type III domains 6–8 of mouse collagen XII was amplified by PCR using primers that introduced a Nhel restriction site at the 5′-end and a BamHI site at the 3′-end (M920, forward, AATGCTACGTTGAAAAAGCTGCTCACC; and M921, reverse, 2TTTGGATCTTAAAGAGATAGAATGGTGCAC). The cDNA was cloned into a modified pET vector (EMD Biosciences) carrying a His₆ tag and a thrombin cleavage site. Upon transformation with the recombinant plasmid, Escherichia coli cells (BL21) were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and grown for 16 h at 30 °C. The cells were harvested and resuspended in Tris-buffered saline, pH 8.0, containing 7 M urea. The bacteria were sonicated followed by removal of insoluble cell debris by centrifugation. After a 2-fold dilution with H₂O the supernatant was applied to a nickel-chelating Sepharose column and eluted with a buffer containing 40–80 mM imidazole. Following removal of urea by dialysis, thrombin cleavage was performed overnight at room temperature (5 mM CaCl₂, 1 unit/mg thrombin, Sigma), and the cleaved His₆ tag was removed by loading the solution again to a nickel-chelating Sepharose column.

Interaction between Venom Proteins and Fibroblast Membranes

Cell Membrane Preparations—Human HS68 fibroblasts grown to 80% confluence in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum were detached from tissue culture plates by a rubber policeman, suspended in phosphate-buffered saline (PBS), and centrifuged. The cell pellet was resuspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ and homogenized with a Polytron for 30 s at 0 °C. The homogenate
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was centrifuged at 250 × g for 5 min at 0 °C, and the supernatant was collected. The pellet was resuspended and centrifuged again. The supernatant was combined with the other supernatant and centrifuged at 1,500 × g for 10 min. The pellet was resuspended and centrifuged at 82,000 × g for 60 min. The resulting pellet representing cell membranes was resuspended in PBS and centrifuged at 1,500 g. The supernatant was combined with the other supernatant, and the solubilized membrane proteins were obtained in the supernatant.

Cy-5 Labeling of Cell Membrane Proteins—50 μg of the fibroblast cell membrane protein preparation was incubated with 400 pmol of Cy-5 dye (GE Healthcare) in 0.1 m sodium carbonate, pH 9.3, for 30 min on ice. The reaction was quenched by adding 10 mM lysine, and the labeled proteins were separated from the reaction mixture by a gel filtration spin column (GE Healthcare).

Biotinylation of C. atrox Venom Proteins—3 mg of C. atrox venom (Miami Serpentarium, Punta Gorda, FL) was mixed with 150 μl of 10 mM biotin (Pierce) in PBS. Following incubation for 2 h on ice, the mixture was dialyzed for 4 h at 4 °C against PBS and stored at 4 °C until use.

Interaction of Biotinylated Venom Proteins and Cy-5-labeled Cell Membrane Proteins—100 μg of biotinylated venom proteins in PBS buffer was incubated with 100 μg of the Cy-5-labeled fibroblast cell membrane protein preparation for 16 h at 4 °C with constant mixing. To stabilize interacting proteins the incubation mixture was incubated with 1 mM bis(sulfosuccinimidyl)suberate (Pierce) for 2 h in ice. The reaction mixture was then dialyzed against PBS for 8 h at 4 °C followed by addition of Nonidet P-40 to a final concentration of 0.5% and NaCl to a final concentration of 0.5 M. This was then incubated with streptavidin beads for 16 h at 4 °C with constant mixing followed by four washes with PBS binding buffer followed by elution with binding buffer containing 100 mM dithiothreitol. The eluted proteins were electrophoresed on a 10% SDS-PAGE gel (21) followed by detection of Cy-5-labeled/biotinylated/cross-linked protein complexes using a Bio-Rad FX laser scanner using a 635 nm laser with a 690-nm band pass filter.

Identification of Venom-Cell Membrane-associated Protein Complexes

For protein identification, protein bands visualized in the gel were excised, destained, and then in-gel digested with trypsin (22). Tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry with a Finnigan LCQ Deca ion trap mass spectrometer system (Finnigan, San Jose, CA) with a Protea nanospray ion source interfaced to a self-packed 8-cm (75-μm-inner diameter) Phenomenex Jupiter 10-μm C18 reverse phase capillary column (Phenomenex, Torrence, CA). Volumes of 0.5–5 μl of extract were injected, and the peptides were eluted from the column by an acetonitrile, 0.1 M acetic acid gradient at a flow rate of 0.25 μl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan spectra to determine peptide M, and product ion spectra to determine amino acid sequence in sequential scans. The data were analyzed by data base searching using the Sequest search algorithm.

Surface Plasmon Resonance Assays—Protein-protein interactions were assayed by surface plasmon resonance with a BIAcore™ 3000 system. Collagen XII or collagen XIV was covalently immobilized on the BIAcore CM-5 sensorchip (carboxylated dextran matrix) according to the instructions of the manufacturer. Briefly the CM-5 chip was activated with a 1:1 mixture of 75 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 11.5 mg/ml N-hydroxysuccinimide for 7 min. Collagen XII or collagen XIV (200 μg/ml in 10 mM sodium citrate, pH 4.5) was injected over an activated CM-5 chip for 10 min at a flow rate of 10 μl/min at 25 °C. Remaining active groups on the matrix were blocked with 1 mM ethanolamine HCl at pH 8.5. Immobilization of collagen XII and collagen XIV on CM-5 sensorchip resulted in average surface concentrations of 13 and 15 ng/mm², respectively. Protein solutions of jararhagin (31.5–2000 nm) and A/C (2.34–300 nm) were prepared in 10 mM HEPES, pH 7.4, 150 mM sodium chloride, 3.4 mM EDTA, 0.005% surfactant P20 (v/v) and injected at a flow rate of 50 μl/min.

Full-length matrins 1, 2, 3, and 4 or matrin VWA domains 3A1, 4A1, and 4A2 were immobilized by injecting the protein (200 μg/ml in 10 mM sodium citrate, pH 4.5) over activated CM-5 chips for 7 min at a flow rate of 10 μl/min at 25 °C, resulting in surface concentrations of 3.18, 3.41, 5.3, 9.35, 6.5, 6.15, and 5.79 ng/mm², respectively. Protein solutions of the recombinant protein A/C (2.81–180 nm) were prepared in 10 mM HEPES, pH 7.4, 150 mM sodium chloride, 3.4 mM EDTA, 0.005% (v/v) surfactant P20 and injected at a flow rate of 50 μl/min.

Collagen XII fibronectin type III domains 6–8 (50 μg/ml in 10 mM sodium acetate, pH 5.5) or matrins 4 EGF domains (10 μg/ml in 10 mM sodium acetate, pH 5.5) were immobilized by injecting the protein solution over activated CM-5 chips for 7 min at a flow rate of 5 μl/min at 25 °C, resulting in surface concentrations of 0.9 and 0.67 ng/mm², respectively. Asolution of 5 μM jararhagin was prepared in 10 mM HEPES, pH 7.4, 150 mM sodium chloride, 3.4 mM EDTA, 0.005% (v/v) surfactant P20 and injected at a flow rate of 30 μl/min.

The nonlinear fitting of association and dissociation curves according to a 1:1 model was used for the calculation of kinetic constants (Biaevaluation software version 3.1). Individual experiments were performed a minimum of six times.

Cleavage of Collagen XII and Collagen XIV by Jararhagin and Identification of Sites of Action

4 μg of collagen XII or XIV was incubated with 0.4 μg of jararhagin for 0, 15, 30, 60, and 120 min at 37 °C in 50 mM Tris-Cl, pH 7.5, 2 mM CaCl₂ as were controls of collagen and jararhagin alone. Reactions were stopped by addition of 20 mM EDTA and freezing prior to electrophoresis on 4–15% SDS-polyacrylamide gels. Following electrophoresis the gels were stained overnight with SYPRO Ruby (Bio-Rad). Gels were scanned with a Bio-Rad FX imager, and molecular weights were estimated from the markers using Bio-Rad Quantity One software.
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Cleavage of Collagen XII in Cultured Fibroblasts by Jararhagin

Fibroblasts were isolated from newborn mouse skin according to established procedures (23) and cultivated in Dulbecco’s modified Eagle’s medium/F12 with GlutamaxTM (Invitrogen) supplemented with 10% fetal calf serum, 250 μM l-ascorbic acid, and 450 μM l-ascorbic acid 2-phosphate. Subconfluent cells were transferred to serum-free medium for 12 h followed by addition of jararhagin to a final concentration of 117 or 233 nM and incubated for 1.5 or 4 h at 37°C. The conditioned medium was supplemented with 20 mM EDTA and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Roche Applied Science), centrifuged at low speed to remove cell debris, and used for Western blot analysis. For detection of collagen XII affinity-purified polyclonal rabbit anti-collagen XII (24) was used.

Cleavage of Matrilin 3 and Matrilin 4 by Jararhagin

Due to the lack of sufficient amounts of matrilin 1 and the lack of observed binding of A/C to matrilin 2, proteolysis experiments were performed with only matrilins 3 and 4. In these experiments 3 μg of matrilin 3 or 2.5 μg of matrilin 4 was incubated with 0.3 μg and 0.25 μg of jararhagin, respectively, for 180 min at 37°C in 50 mM Tris-Cl, pH 7.5, 2 mM CaCl2 as were controls of matrilins and jararhagin alone. Reactions were stopped by freezing at −80°C before electrophoresis on 8–16% SDS-polyacrylamide gels that were stained overnight with SYPRO Ruby (Bio-Rad). Gels were scanned with a Bio-Rad FX imager, and molecular weights were estimated using Bio-Rad Quantity One software. As in the case of the collagens, the C termini of the proteolysis products were estimated based on their identified N termini, their molecular masses from gel electrophoresis, and their published sequences. The figure presented is representative of a minimum of three such experiments.

RESULTS

Identification of C. atrox Venom Protein-Fibroblast Membrane-associated Protein Complexes—The concept behind this experiment was to attempt to identify novel venom protein-cell surface-associated protein interactions. As shown in supplemental Fig. 1, the approach was to label venom proteins with biotin and fibroblast cell surface-associated proteins with the fluorescent dye Cy-5. Following mixture of the two preparations protein-protein interactions were stabilized by chemical cross-linking. The labels on these proteins were then exploited such that when the mixture was passed through a streptavidin bead column only proteins and protein complexes with biotin

To identify sites of digestion, 20 μg of collagen was incubated with 2 μg of jararhagin as described above, and proteins were transferred to polyvinylidene difluoride membrane and stained with Coomassie Blue. For blotting, the gel was placed in a Transphor apparatus (GE Healthcare), and proteins were transferred at 0.4 A (3.17 mA/cm2) for 1.5 h at a maximum of 100 V to 0.2-μm pore polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) using 25 mM Tris, 192 mM glycin at 10°C. After staining the membrane with Coomassie Blue the membrane was scanned using a Bio-Rad GS-800 scanner, and bands of interest were excised for N-terminal amino acid sequencing. After washing the membrane slices with 20% methanol, the bands were sequenced in an Applied Biosystems 494 Procise sequencer using the manufacturer’s pulsed liquid polyvinylidene difluoride cycles.

The C termini of the digested collagens were localized by estimation based on their identified N termini, the estimated molecular mass of the fragment from gel electrophoresis, and the published sequences of these proteins using the ExPASy protein Compute pl/Mw tool. The figure presented is representative of a minimum of three such experiments.

TABLE 1

Kinetic evaluation of the interaction of jararhagin and A/C (recombinant cysteine-rich domain of PIII SVMP atrolysin A) with collagens XII and XIV immobilized on CM-5 sensorchip in the BIAcore 3000 system

| Analyte | Ligand on chip | Kd (nM) | kₚ (× 10⁷ M⁻¹ s⁻¹) | kₐ (× 10⁻⁵ s⁻¹) |
|---------|----------------|---------|---------------------|-----------------|
| Jararhagin | Collagen XII | 285 | 0.0218 | 6.2 |
| Jararhagin | Collagen XIV | 254 | 0.00754 | 1.92 |
| A/C | Collagen XII | 5.31 | 0.211 | 1.12 |
| A/C | Collagen XIV | 9.37 | 0.114 | 1.07 |

FIGURE 1. Interaction of jararhagin and the cysteine-rich domain of atrolysin A (A/C) to collagens XII and XIV in the BIAcore 3000 system. Jararhagin (0–2000 nM) and A/C (0–300 nM) were injected over immobilized collagens at a flow rate of 50 μl/min. A, jararhagin over collagen XII; B, jararhagin over collagen XIV; C, A/C over collagen XII; D, A/C over collagen XIV.

Identification of C. atrox Venom Protein-Fibroblast Membrane-associated Protein Complexes—The concept behind this experiment was to attempt to identify novel venom protein-cell surface-associated protein interactions. As shown in supplemental Fig. 1, the approach was to label venom proteins with biotin and fibroblast cell surface-associated proteins with the fluorescent dye Cy-5. Following mixture of the two preparations protein-protein interactions were stabilized by chemical cross-linking. The labels on these proteins were then exploited such that when the mixture was passed through a streptavidin bead column only proteins and protein complexes with biotin
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**TABLE 2**

Kinetic evaluation of the interaction of A/C with full-length matrilins and matrilin VWA domains immobilized on CM-5 sensorchip in the BIAcore 3000 system

| Ligand on chip       | $K_D$ | $k_	ext{on}$ | $k_	ext{off}$ |
|----------------------|-------|---------------|---------------|
| Matrilin 1           | 4.64  | 1.61          | 4.48          |
| Matrilin 2           | N.D.  |               |               |
| Matrilin 3           | 3.65  | 1.53          | 5.58          |
| Matrilin 3 A1 domain | 2.58  | 1.40          | 3.63          |
| Matrilin 4           | 35.9  | 0.19          | 6.77          |
| Matrilin 4 A1 domain | 22.9  | 0.14          | 3.08          |
| Matrilin 4 A2 domain | 14.8  | 0.16          | 2.29          |

tags (via labeled venom proteins) would be retained. Following elution of the column and electrophoresis via SDS-PAGE detection of Cy-5-tagged protein would be indicative of cross-linked protein complexes containing both cell membrane-associated and venom proteins (supplemental Fig. 1). All fluorescent bands were subjected to liquid chromatography-tandem mass spectrometry analysis, and the band indicated with the arrow in supplemental Fig. 1 contained peptides derived from both the *C. atrox* PIII SVMP, catrocollastatin ((I/L)PCAPE-DVK), and the FACIT collagen XIV (TNQ(L/I)(I/L)(L/I)QNT).

Interaction of the PIII SVMP Jararhagin and the Recombinant Cys-rich Domain from the PIII SVMP Atrolysin A with Collagens XII and XIV—Our aim with these studies was to investigate further the interaction of class PIII SVMPs with the FACIT collagens and to localize the interaction domain on the PIII SVMPs. For this purpose, jararhagin, a PIII SVMP from *B. jararaca* venom, and a recombinant cysteine-rich domain (A/C) of atrolysin A from *C. atrox* venom were assayed to assess their ability to bind to collagens XII and XIV, both containing VWA domains, using surface plasmon resonance. As observed in Fig. 1, both jararhagin (Fig. 1, A and B) and the recombinant cysteine-rich domain A/C (Fig. 1, C and D) were capable of interacting with immobilized collagens XII and XIV, respectively, in a concentration-dependent manner. The kinetics of these interactions are shown in Table 1. Interestingly the recombinant PIII cysteine-rich domain A/C demonstrated significantly stronger interaction to collagens XII and XIV than did the full-length SVMP jararhagin. In another interaction assay using surface plasmon resonance jararhagin showed no binding to recombinant collagen XII fibronectin type III domains 6–8 indicating that the interactions with FACIT collagens are mediated by the VWA domains present in collagens XII and XIV (not shown).

**Interaction of the Recombinant Cysteine-rich Domain of Atrolysin A (A/C) with Full-length Matrilins**—To extend the possibility that other PII SVMPs containing cysteine-rich domains could bind to VWA domain-containing matrix proteins we assayed the ability of the recombinant *C. atrox* A/C protein from atrolysin A to bind to matrilins 1–4. As seen in Fig. 2, A/C was capable of binding to matrilins 1, 3, and 4 in a concentration-dependent manner. No binding was observed between A/C and matrilin 2. The kinetics of these interactions are shown in Table 2. The binding of A/C to matrilins 1, 3, and 4 was all in the nanomolar range; however, the interaction with matrilins 1 and 3 was ~8 times stronger than with matrilin 4. No binding was detected when we tested the interaction of jararhagin with a recombinant protein corresponding to the matrilin 4 EGF domains (not shown).

**Interaction of the Recombinant Cysteine-rich Domain of Atrolysin A (A/C) with Recombinant Matrilin 3 and 4 VWA Domains**—Fig. 3 shows the results of the interaction of A/C with the available recombinant matrilin 3 VWA1 domain and the matrilin 4 VWA1 and VWA2 domains using surface plasmon resonance. A/C bound to the three matrilin VWA domains in the nanomolar $K_D$ range. The binding to the two matrilin 4 VWA domains was with similar $K_D$ values; however, they were notably less than the $K_D$ value for A/C binding to the matrilin 3 VWA1 domain. These values reflect the binding observations for A/C with the full-length matrilins in that $K_D$ values for A/C binding to matrilins 1 and 3 were significantly lower than that for matrilin 4.

**Proteolysis of Collagens XII and XIV and Matrilins 3 and 4**—Proteolysis of extracellular matrix is considered to be a critical factor in the production of hemorrhage by certain SVMPs, hence the ability of jararhagin to cleave collagens XII and XIV and matrilins 3 and 4 was investigated. As seen in Figs. 4, A and B, collagens XII and XIV, respectively, showed specific, limited proteolysis by jararhagin over time. Cleavage products from the collagens following incubation with jararhagin when subjected
to N-terminal sequence analysis indicated that the sites of cleavage in the collagens were localized to regions within or proximal to VWA domains (Table 3 and Fig. 5). In the case of collagen XII cleavages in the VWA4 domain gave rise to one N-terminal fragment, band B, with its C terminus localized in the VWA4 domain and four bands (C–F) with their N termini localized at or adjacent to the VWA4 domain and their C termini localized to the C-terminal region of the intact protein. For collagen XIV, four bands (A–D) had N termini identified adjacent to the VWA1 domain with C termini localized to regions in or adjacent to the VWA2 domain (Table 3 and Fig. 5). One band, E, had proteins with N termini identified in the VWA2 domain and immediately C-terminal to the VWA2 domain with their C termini localized to the C-terminal collagen 2 domain. Band F contained proteins with N termini identified immediately N-terminal to the VWA1 domain with C termini localized to the C-terminal collagen 2 domain and the VWA2 domain in the N-terminal region of the fibronectin type III domain 2 (C-terminal to the VWA1 domain). Band G also contained proteins with N termini identified adjacent to the N terminus of the VWA1 domain with C termini localized to the area immediately C-terminal to the VWA1 domain. These data corroborate our findings that PIII SVMPs such as jararhagin interact with these FACIT collagens by virtue of their cysteine-rich domain binding to VWA domains in the collagens and in doing so promote proteolysis at sites on or nearby the VWA domain.

**FIGURE 3. Interaction of the cysteine-rich domain of atrolysin A (A/C) to matrilin VWA domains in the BIAcore 3000 system.** A/C (0–90 nm) was injected over immobilized matrilin 3A1 (A), matrilin 4A1 (B), and matrilin 4A2 (C) at a flow rate of 50 µl/min.

**FIGURE 4. Cleavage of collagens by jararhagin.** A, collagen XII cleavage by jararhagin: lane 1, molecular mass markers; lane 2, collagen XII incubated without jararhagin for 60 min; lanes 3–6, collagen XII incubated with jararhagin for 0 min (lane 3), 15 min (lane 4), 30 min (lane 5), and 60 min (lane 6). B, collagen XIV cleavage by jararhagin: lane 1, molecular mass markers; lane 2, collagen XIV incubated without jararhagin for 120 min; lane 3, jararhagin incubated for 120 min; lanes 4–8, collagen XIV incubated with jararhagin for 0 min (lane 4), 15 min (lane 5), 30 min (lane 6), 60 min (lane 7), and 120 min (lane 8). Numbers on the left indicate molecular mass marker mobility. Letters and arrows on the right indicate protein bands submitted to N-terminal sequence analysis.
Confirming the functional significance of the proteolytic activity of jararhagin on FACIT collagens in a matrix, jararhagin was also able to cleave collagen XII in the extracellular matrix generated by intact fibroblasts. Fig. 6 shows the degradation products detected by Western blot analysis, using an anti-collagen XII antibody, of primary newborn mouse fibroblast cultures incubated with jararhagin indicating that collagen XII was cleaved in a time- and concentration-dependent fashion.

When jararhagin was incubated with matrilins 3 and 4 both showed evidence of proteolysis by jararhagin (Fig. 7). Incubation of jararhagin with matrilin 3 showed several products; four of these yielded significant N-terminal sequence information (Table 3 and Fig. 7A). Band A contained three proteins with N termini located immediately N-terminal to the VWA1 domain with C termini localized to the C-terminal region of the protein (Fig. 5). Bands B and C contained proteins with their nascent N termini within the VWA domain and the C termini at the C-terminal region of the protein. Band D was identified to contain proteins with their N termini at the C terminus of the VWA domain and with their C termini localized to the C-terminal region of the protein. As in the case with collagens XII and XIV, jararhagin cleavage of matrilin 3 was observed at or adjacent to the VWA domain suggesting localized, specific interaction with that domain. The proteolysis of matrilin 4 by jararhagin is shown in Fig. 7B. Clearly there was digestion of matrilin 4 by jararhagin; however, the five bands submitted to Edman analysis did not yield interpretable data. Nevertheless one can speculate that the primary cleavage product, band D, at 50 kDa, is the result of a loss of ~20 kDa from either the N or C terminus that would place the cleavage site within or adjacent to either the VWA1 or VWA2 domain, respectively.

### DISCUSSION

Over the past several years there has been mounting evidence suggesting the interaction of members of the Reprolysin family of metalloproteinases with proteins that contain von Willbrand factor A domains. The PIII SVMP jararhagin has been demonstrated to bind to and cleave VWF and the integrin α2β1

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**Table 3**

| Protein band | Molecular weight of gel band | Observed N-terminal sequence (after arrow) | Domain of N-terminal cleavage site | Domain of C-terminal cleavage site |
|--------------|------------------------------|------------------------------------------|-----------------------------------|-----------------------------------|
| Collagen XII |                             |                                          |                                   |                                   |
| A            | 170,278                      | M\(^{190}\)ECLTRA (N terminus of undigested starting material) | N terminus of the small splice variant, after signal peptide, without the collagenous domain | Intact C terminus of protein       |
| B            | 134,948                      | MECLTRA                                  | Same as band A                     | VWA4 domain                       |
| C            | 85,200                       | PSP \[^{202}\]AQG                        | Fibronectin type III repeat 14     | Intact C terminus of protein       |
| D            | 43,128                       | ALA \[^{230}\]LGA                        | VWA4 domain                        | Intact C terminus of protein       |
| E            | 39,702                       | LGA \[^{234}\]QN                         |                                     | Immediately carboxyl to the thrombospondin domain |
| F            | 32,803                       | GKA \[^{260}\]LTF                       | VWA4 domain                        | Intact C terminus of protein       |
| Collagen XIV |                             |                                          |                                   |                                   |
| A            | 136,874                      | KAS \[^{35}\]ALA                         | VWA1-fibronectin type III repeat 2 interdomain region | C-terminal NC2 domain             |
| B            | 110,818                      | ASA \[^{35}\]LAT                         |                                     | Thrombospondin domain             |
| C            | 98,001                       | ESA                                         | VWA2 domain                        | C-terminal COL2 domain            |
| D            | 84,658                       | VSA                                         | VWA2-thrombospondin interdomain region | C-terminal COL2 domain            |
| E            | 38,375                       | YSE \[^{117}\]LVS                       | VWA2 domain                        |                                    |
| F            | 26,248                       | PTX \[^{127}\]YH                         | Thrombospondin domain              |                                    |
| G            | 23,173                       | KGG \[^{139}\]ENK                       | Fibronectin type III repeat 1-VWA1 interdomain region |                                    |
| Matrilin 3  |                             |                                          |                                   |                                   |
| A            | 40,960                       | LSA \[^{55}\]LAT                         | N-terminal region of protein       | C-terminal region of protein       |
| B            | 32,970                       | TGT \[^{125}\]VCX                       | VWA1 domain                        | C-terminal region of protein       |
| C            | 27,720                       | EEA \[^{116}\]FTV                       | VWA1 domain                        | C-terminal region of protein       |
| D            | 25,250                       | IEN \[^{25}\]LA                          | VWA1 domain                        | C-terminal region of protein       |
In both cases binding to these proteins resulted in proteolytic cleavage of the protein. However, the precise sites of digestion, and presumably interaction, were not determined.

Another member of the Reprolysin family, ADAMTS13, has been demonstrated to function in normal hemostasis by binding to VWF and proteolytically cleaving it to modulate the size of the circulating von Willebrand factor multimers (25). ADAMTS13 cleaves the Tyr1605-Met1606 bond in the VWA2 domain of VWF to produce smaller multimeric VWF from the ultralarge VWF (26). In recent studies using a variety of recombinant forms of VWA domains, the site of interaction of ADAMTS13 on VWF, as well as proteolysis, has been delimited to the VWA2 domain (27–29).

Several groups have investigated the regions of ADAMTS13 that are involved in VWF interaction. Soejima et al. (30) demonstrated that the cysteine-rich/spacer domains of ADAMTS13 are critical for effective proteolysis of VWF. Similarly Sadler and co-workers (31) have shown that truncation of...
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ADAMTS13 after the first thrombospondin type 1 repeat greatly reduced binding to VWF, whereas truncation after the spacer domain did not significantly affect binding thus implicating the cysteine-rich and/or the spacer domain as playing a major role in VWF binding.

Previous studies with both PIII SVMPs and ADAMTS13 have suggested that the site in these proteins involved in VWA domain interaction may reside in the disintegrin-like/cysteine-rich domain. Three PIII SVMPs, jararhagin from B. jararaca venom, atrolysin A from C. atrox venom, and kaouthiagin from Naja kaouthia venom, have been demonstrated to bind and/or proteolytically cleave VWF (9, 10, 32). In the case of kaouthiagin, the site of cleavage in VWF was identified at Pro709, Asp709 in the C-terminal hinge region of the VWA1 domain (32). This cleavage yields two dimeric VWF fragments of 350 and 220 kDa that are incapable of promoting platelet aggregation (32).

As for which of the PIII domain(s) is involved in VWF binding, less is known compared with ADAMTS13. However, only the PIII SVMPs have been demonstrated to bind VWF thereby suggesting a role for their distinguishing disintegrin-like/cysteine-rich domains. The first evidence that the cysteine-rich domain of PIII SVMPs may play a functional role was presented by Jia et al. (7, 33) who showed that the recombinant disintegrin-like/cysteine-rich domain of the PIII SVMP atrolysin A as well as the recombinant cysteine-rich domain alone could inhibit collagen-stimulated platelet aggregation. These studies suggested a functional role of the cysteine-rich domain with the platelet collagen receptor α2β1, which possesses a VWA domain. Zigrino et al. (8) substantiated these results by demonstrating that the PIII SVMP jararhagin bound to the α2β1 integrin in a non-metal-dependent fashion. Furthermore Serrano et al. (10) recently reported that the recombinant cysteine-rich domain of atrolysin A could bind to VWF and block its interaction with collagen, again providing an indication of a functional role for the cysteine-rich domain of PIII SVMPs. This was extended by the work of Kamiguti et al. (34) who showed that synthetic peptides from the cysteine-rich domain of atrolysin A and jararhagin could block collagen-stimulated platelet aggregation and α2β1-mediated cell adhesion to collagen. Thus, there are cumulative data that certain members of the Reprolysin family, such as ADAMTS13 and the PIII SVMPs, could target proteins containing VWA domains and effect proteolytic cleavage of the proteins.

To assess the possibility of other novel venom-cell surface protein interactions we launched a discovery approach experiment to detect venom protein-cell surface protein interactions. As described above, we were able to detect an interaction between the PIII SVMP catrocollastatin and collagen XIV. This interaction was localized to the cysteine-rich domain of catrocollastatin using a recombinant protein representing this domain of atrolysin A. Collagen XIV is a member of the FACIT collagens, a group of diverse collagens that function as molecular bridges in extracellular matrices (35). It is comprised of several domain types, including seven fibronectin type III repeat domains, two VWA domains, one thrombospondin domain, two collagen domains, and two non-collagenous (NC) domains (36). Collagen XIV is thought to play a role in matrix stabilization by interacting with collagen fibril surfaces through their NC1 domains (37). As we have shown PIII SVMPs can bind to collagen XIV via its cysteine-rich domain and cleave the collagen at sites on or near the VWA1 and -2 domains suggesting that the binding site on collagen XIV for the cysteine-rich domain in the PIII SVMPs is within the VWA domains.

To determine whether the interaction of matrix proteins containing VWA domains with PIII SVMPs is a general phenomenon, we examined the ability of three other such proteins to support cysteine-rich domain-mediated PIII SVMP interactions. Collagen XII is also a FACIT collagen with four VWA domains (15). Using the short splice variant of collagen XII molecule lacking the first two VWA domains we demonstrated cysteine-rich domain-mediated binding to collagen XII with subsequent proteolytic cleavages localized in the VWA4 domain, again indicating PIII SVMP cysteine-rich domain interaction with VWA domains. Moreover, the interaction and cleavage of collagen XII by jararhagin was demonstrated to occur in matrix produced by cultured fibroblasts pointing to the importance of this extracellular matrix component as a target for jararhagin. The fact that jararhagin was not able to bind to a protein comprised of the fibronectin type III domains 6–8 of collagen XII, i.e. lacking the VWA domains, confirmed the role of the VWA domain in the recognition of collagen XII by jararhagin.

Another family of extracellular matrix proteins that contain VWA domains is the matrilins (16). This family is comprised of four members that are modular, multisubunit proteins predominantly expressed in cartilage. Like the FACIT collagens they appear to bridge other matrix proteins such as fibrillar collagens and aggrecan. Matrilins 1, 2, and 4 possess two VWA domains separated by EGF-like repeats, whereas matrilin 3 has only one VWA domain followed by EGF-like repeats. The matrilins undergo oligomerization mediated by their VWA domains. Using full-length matrilins 1–4 we were able to demonstrate cysteine-rich domain binding with all but matrilin 2. Furthermore using recombinant matrilin 3 VWA1 and matrilin 4 VWA1 and VWA2 domains we also demonstrated their ability to interact with PIII SVMP cysteine-rich domain. The lack of binding to matrilin 2 could be explained by a blocking of the binding site due to a strong self-interaction of both VWA domains as has been shown by electron microscopy (38). We also assayed whether the PIII SVMP jararhagin could proteolytically cleave matrilins 3 and 4, and as was observed with collagen XII and XIV cleavage was detected at or near the single VWA domain in the case of matrilin 3. Confirming that the interactions of the PIII SVMPs to the matrilins are mediated by the VWA domain, no binding was observed by surface plasmon resonance when jararhagin was injected over a recombinant form of matrilin 4 lacking the VWA domains.

Recently the crystal structure of the PIII SVMP VAP1 was solved (39). Examination of the structure indicated that the RGD loop-like region of the disintegrin-like domain of the PIII was not exposed for protein-protein interaction, whereas the cysteine-rich domain was available for such interactions. This corroborates our data that the cysteine-rich domain is both capable of and critical for protein-protein interactions. One can envision situations where the disintegrin-like/cysteine-rich domains have been proteolytically processed from the full-
length PIII, and the disintegrin-like domain may support protein-protein interactions, but based on the structure, this is not likely the case in the full-length protein with the disintegrin-like/cysteine-rich domains presented in the context of the metalloproteinase domain. Interestingly in another recent study it was shown that oxiagin, a PIII metalloproteinase isolated from the venom of *Naja oxiana*, was able to inhibit the classical pathway of the complement system by preventing the interaction of component C2 (without its inactivation) with immobilized component C4b. Complement factor C2 contains a VWA domain; hence one can speculate that oxiagin interacts with C2 VWA domain via its cysteine-rich domain thus preventing the binding of C2 and C4b that is known to be dependent on Mg²⁺-binding sites within the VWA domain.

An inspection of the alignment of the amino acid sequences of several PIII SVMPs, ADAMs, and ADAMTS13 disintegrin-like and cysteine-rich domains shows that ADAMTS13 structure is rather divergent from that of the SVMPs and ADAMs having two sequence insertions in the disintegrin-like domain and one in the cysteine-rich domain (supplemental Fig. 2). Furthermore the cysteinyl residue pattern of ADAMTS13 is also somewhat less conserved in both the disintegrin-like and cysteine-rich domains compared with the SVMPs and the ADAMs proper. As such it is likely that the tertiary structure of the disintegrin-like/cysteine-rich domains of ADAMTS13 is somewhat different from that of either the SVMPs or the ADAMs. Therefore, one can speculate that the motifs present in the cysteine-rich domains of the SVMPs and ADAMTSs that play a role in VWA domain interaction are based on chemically similar residues topographically displayed in such a manner that, despite dissimilar structures, effective ligation between those motifs in the cysteine-rich domains of SVMPs and ADAMTS13 and VWA domains can occur.

Thus, from the data generated in this investigation we can state that the cysteine-rich domain of PIII SVMPs functions to target the proteinase to these extracellular matrix components by binding to their VWA domains followed by proteolytic cleavage at or near the VWA domain. In the case of VWF, interaction with PIII SVMPs and ADAMTS13 gives rise to functionally impaired VWF in the case of the former and a non-pathological, smaller multimer of VWF in the latter. Given the important role FACIT collagens XII and XIV play in stabilizing fibrillar collagen structure and the function of matrilins as adaptor proteins bridging matrix proteins it is straightforward to suggest that targeting and cleaving these proteins by the PIII SVMPs could play an important role in their most striking pathological feature, the production of local and systemic hemorrhage (2, 3). Previously we have shown that the hemorrhagic SVMPs were potent agents of capillary basement membrane degradation that leads to openings in the capillary wall allowing the escape of capillary contents into the stroma (5, 6). The basement membranes surrounding the capillaries are in contact with and presumably stabilized by the surrounding stromal matrix, and cleaving proteins involved in stabilizing stromal matrix could promote or synergize the destabilization of basement membranes and contribute to the overall hemorrhagic effect. Studies to demonstrate this concept are currently underway in our laboratory.

This investigation serves to underscore a general principle of toxinology that toxins are simple variants of normal proteins, as evidenced by the PIII SVMPs and the ADAMs, where the pathological consequences of the toxins are due to their presence at the wrong place or wrong time or at an inappropriate concentration in the prey. Furthermore, based on these studies, one can hypothesize that the presence of VWA domains in matrix proteins, as well as other families of proteins, may serve a broader function. The role of VWA domains as targeting sites for ADAM proteaseinase for subsequent proteolytic processing of the molecule with the result of altering its biological function in accord with a particular cellular process may be more widespread than the example of VWF and ADAMTS13 and thus suggests a potential interesting area of research for further study.

REFERENCES

1. Oweny, C. L., Bjarnason, J., and Tu, A. T. (1978) *Am. J. Pathol.* 93, 201–218.
2. Ohsaka, A., Ikazawa, H., Kondo, H., Kondo, S., and Uchida, N. (1960) *Br. J. Exp. Pathol.* 41, 475–486.
3. Bjarnason, J. B., and Fox, J. W. (1995) *Methods Enzymol.* 248, 345–368.
4. Fox, J. W., and Serrano, S. M. T. (2005) *Toxicon* 45, 969–985.
5. Baramova, E. N., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1989) *Arch. Biochem. Biophys.* 275, 63–71.
6. Shannon, J. D., Baramova, E. N., Bjarnason, J. B., and Fox, J. W. (1989) *J. Biol. Chem.* 264, 11575–11583.
7. Jia, L. G., Wang, X. M., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (2000) *Arch. Biochem. Biophys.* 373, 281–286.
8. Zigirino, P., Kamiguti, A. S., Eble, J., Drescher, C., Nischt, R., Fox, J. W., and Mauch, C. (2002) *J. Biol. Chem.* 277, 40528–40535.
9. Kamiguti, A. S., Hay, C. R., Theakston, R. D., and Zuzel, M. (1996) *Toxicon* 34, 627–642.
10. Serrano, S. M. T., Jia, L. G., Wang, D., Shannon, J. D., and Fox, J. W. (2005) *Biochem. J.* 391, 69–76.
11. Whittaker, C. A., and Hynes, R. O. (2002) *Mol. Biol. Cell.* 13, 3369–3387.
12. Gordon, M. K., Gerecke, D. R., and Olsen, B. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 6040–6044.
13. Gordon, M. K., Castagnola, P., Dublet, B., Linsenmayer, T. F., Van der Rest, M., Mayne, R., and Olsen, B. R. (1991) *Eur. J. Biochem.* 201, 333–338.
14. Gerecke, D. R., Foley, J. W., Castagnola, P., Gennari, M., Dublet, B., Cancedda, R., Linsenmayer, T. F., Van der Rest, M., Olsen, B. R., and Gordon, M. K. (1993) *J. Biol. Chem.* 268, 12177–12184.
15. Bohme, K., Li, Y., Oh, P. S., and Olsen, B. R. (1995) *Dev. Dyn.* 204, 432–445.
16. Wagener, R., Ehlen, Harald, W. A., Ko, Y.-F., Kobbe, B., Mann, H. H., Sengle, G., and Paulsson, M. (2005) *FEBS Lett.* 579, 3323–3329.
17. Paine, M. J., Desmond, H. P., Theakston, R. D., and Crampston, J. M. (1992) *J. Biol. Chem.* 267, 22869–22876.
18. Nishiyama, Y., McDonough, A. M., Bruns, R. R., and Burgeson, R. E. (1994) *J. Biol. Chem.* 269, 28193–28199.
19. Kohfeldt, E., Maurer, P., Vannahme, C., and Timpl, R. (1997) *FEBS Lett.* 414, 557–561.
20. Laemmli, U. K. (1970) *Nature* 227, 680–685.
21. Hanna, S. L., Sherman, N. E., Kinter, M. T., and Goldberg, J. B. (2000) *Microbiology* 146, 281–286.
22. Zhang, Z. G., Bothe, I., Hirche, F., Zweers, M., Gullberg, D., Pfitzer, G., Krieg, T., Eckes, B., and Aumaillé, M. (2006) *J. Cell Sci.* 119, 1886–1895.
23. Veit, G., Hansen, U., Keene, D. R., Bronck, P., Chiquet-Ehrismann, R., Chiquet, M., and Koch, M. (2006) *J. Biol. Chem.* 281, 27461–27470.
24. Rest, M., Mayne, R., and Olsen, B. R. (1991)
25. Zigrino, P., Kamiguti, A. S., Eble, J., Drescher, C., Nischt, R., Fox, J. W., and Mauch, C. (2002) *J. Biol. Chem.* 277, 40528–40535.
26. Furlan, M., Robles, R., and Lammle, B. (1996) *Blood* 96, 1808–1815.
Interaction of SVMPs with VWA Domains

27. Kokame, K., Matsumoto, M., Fujimura, Y., and Miyata, T. (2004) Blood 103, 607–612
28. Nishio, K., Anderson, P. J., Zheng, X. L., and Sadler, J. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10578–10583
29. Zanardelli, S., Crawley, J. T. B., Chan Kwo Chion, C. K. N., Lam, J. K., Preston, J. S., and Lane, D. A. (2006) J. Biol. Chem. 281, 1555–1563
30. Soejima, K., Matsumoto, M., Kokame, K., Yagi, H., Ishizashi, H., Maeda, H., Nozaki, C., Miyata, T., Fujimura, Y., and Nakagaki, T. (2003) Blood 102, 3232–3237
31. Majerus, E. M., Anderson, P. J., and Sadler, J. E. (2005) J. Biol. Chem. 280, 21773–21778
32. Hamako, J., Matsui, T., Nishida, S., Nomura, S., Fujimura, Y., Ito, M., Ozeki, Y., and Titani, K. (1998) Thromb. Haemostasis 80, 499–505
33. Jia, L. G., Wang, X. M., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1997) J. Biol. Chem. 272, 13094–13102
34. Kamiguti, A. S., Gallagher, P., Marcinkiewicz, C., Theakston, R. D. G., Zuzel, M., and Fox, J. W. (2003) FEBS Lett. 549, 129–134
35. Shaw, L. M., and Olsen, B. R. (1991) Trends Biochem. Sci. 16, 191–194
36. Gerecke, D. R., Meng, X., Liu, B., and Birk, D. E. (2003) Matrix Biol. 22, 209–216
37. Eyre, D. (2002) Arthritis Res. 4, 30–35
38. Piecha, D., Muratoglu, S., Mørgelin, M., Hauser, N., Studer, D., Kiss, I., Paulsson, M., and Deák, F. (1999) J. Biol. Chem. 274, 13353–13361
39. Takeda, S., Igarashi, T., Mori, H., and Araki, S. (2006) EMBO J. 25, 2388–2396
40. Shoibonov, B. B., Osipov, A. V., Kryukova, E. V., Zinchenko, A. A., Lakhtin, V. M., Tsetlin, V. I., and Utkin, Y. N. (2005) Mol. Immunol. 42, 1141–1153