Identification of an ADAMTS-4 Cleavage Motif Using Phage Display Leads to the Development of Fluorogenic Peptide Substrates and Reveals Matrilin-3 as a Novel Substrate

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ADAMTS-4 and ADAMTS-5 are aggrecanases responsible for the breakdown of cartilage aggrecan in osteoarthritis. Multiple ADAMTS-4 cleavage sites have been described in several matrix proteins including aggrecan, versican, and brevican, but no concise predictive cleavage motif has been identified for this protease. By screening a 13-mer peptide library with a diversity of 10⁸, we have identified the ADAMTS-4 cleavage motif E-(AVFLMY)-X(0,1)-(RK)-X(2,3)-(ST)-(VYIFWMLA), with Glu representing P1. Several 13-mer peptides containing this motif, including DVQEFRGVTAVIR and HNEFRQRETYMVF, were shown to be substrates for ADAMTS-4. These peptides were found to be specific substrates for ADAMTS-4 as they were not cleaved by ADAMTS-5. Modification of these peptides with donor (6-FAM) and acceptor (QSY-9) molecules resulted in the cleavage by ADAMTS-5. Modification of these peptides with dGlu was tolerable for binding, but not catalysis, whereas substitution of P1 with dPhe precluded both binding and catalysis. Similarly, replacement of Glu with Asp at P1 abolished recognition and cleavage of the peptide. Finally, BLAST results of the ADAMTS-4 cleavage motif identified matrilin-3 as a new substrate for ADAMTS-4. When tested, recombinant ADAMTS-4 effectively cleaved intact matrilin-3 at the predicted motif at Glu⁴³⁵/Ala⁴³⁶ generating two species of 45 and 5 kDa.

The ADAMTS family (A Disintegrin and Metalloprotease with Thrombospondin Motifs) is comprised of 19 members with varied substrate preferences (reviewed in Ref. 1). ADAMTS-2, -3, and -14 have been identified as procollagen N-proteases. ADAMTS-13 maintains hemostasis through the proteolysis of von Willebrand factor following platelet binding. ADAMTS-7 and ADAMTS-12 have been shown to cleave cartilage oligomeric matrix protein. ADAMTS-1, -4, and -5 have been identified as proteoglycanases, cleaving aggrecan, versican, and brevican. ADAMTS-8, -9, -15, -16, and -18 also have been grouped in this family, but their activity toward aggrecan is substantially less compared with the other three members (2). The substrate preferences for the remaining family members have not been conclusively determined. Aggrecanase-mediated degradation of aggrecan, the major aggregating proteoglycan of articular cartilage, is an early and sustained feature of osteoarthritis (OA). ADAMTS-4 and ADAMTS-5 cleave aggrecan at 5 unique sites, all bearing a glutamic acid residue at P1: Glu⁵⁷³/Ala⁵⁷⁴, Glu¹₅₃⁹/Gly¹₅₄₀, Glu¹₇₁⁴/Gly¹₇₁₅, Glu¹₈₁⁹/Ala¹₈₂₀, and Glu¹⁹₁⁹/Leu¹⁹₂₀ (human sequence) (3, 4). Because of their preference for Glu at P1, both ADAMTS-4 and -5 are considered glutamyl endoproteinasises. Whereas ADAMTS-5 is constitutively expressed in human cartilage, ADAMTS-4 is inducible by a number of inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α (5). Gene knockout of ADAMTS-5, but not ADAMTS-4, expression in mice has been shown to be chondroprotective in a surgical mouse model of OA (6, 7), yet in human OA cartilage explants both ADAMTS-4 and ADAMTS-5 mediate aggrecan breakdown (8). Inhibition of ADAMTS-4 and ADAMTS-5 activity may represent a viable option for slowing down the progression of cartilage deterioration in OA.

Alignment of the known sequences flanking the ADAMTS-4 cleavage sites in the proteoglycan substrates, aggrecan, versican, and brevican, led to the proposal of a 24-amino acid consensus motif (9). Not surprisingly, a glutamic acid residue occupied P1 (100% conserved) with P2 occupied by the basic amino acids, Arg or Lys. The authors speculated that activity of ADAMTS family members toward proteoglycan substrates was primarily dictated by an extended 23-amino acid motif N-terminal to the scissile bond, and a short 3-amino acid motif downstream of the site of cleavage. However, unlike the scissile bonds in the aggregating proteoglycans, the site of ADAMTS-4 proteolysis in α₂-macroglobulin (α₂M) is Met⁶⁹⁰/Gly⁶⁹¹ (10), with no requirement for Glu at P1 (10). Yet, P1` to P3` in α₂M, Gly-Arg-Gly, is remarkably similar to downstream sequences in aggrecan and brevican, implying that P` amino acids may be more important in recognition and catalysis than sequences upstream of the scissile bond. ADAMTS-4 has also been shown

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‡The abbreviations used are: ADAMTS, A Disintegrin and Metalloprotease with Thrombospondin Motifs; α₂M, α₂-macroglobulin; OA, osteoarthritis; VWF A, von Willebrand factor A; TBS, Tris-buffered saline; MMP, matrix metalloproteinase; RP-HPLC, reverse phase-high performance liquid chromatography; Ac, acetyl; LC, liquid chromatography; MS, mass spectrometry; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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ADAMTS-4 Cleavage Motif

Expression and Methods—ADAMTS-4—Full-length recombinant human ADAMTS-4 and ADAMTS-5 were cloned and expressed in Drosophila Sf9 cells as previously described (18, 19) and the conditioned media purified by heparin-Sepharose chromatography. The purified ADAMTS-4 preparation was composed almost solely of the 70-kDa active form. Activity was assessed using an enzyme-linked immunosorbent activity assay employing a 36-mer peptide, spanning the site of proteolysis in the interglobular domain of aggrecan, and labeled at the C-terminal with biotin. Upon cleavage of the peptide substrate, the C-terminal proteolytic fragment was detected by BC-3 monoclonal antibody, recognizing the sequence ARGS of no less than 27 amino acids in length (13). Detec-
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Peptide phage display has been demonstrated to be a valid means of determining the consensus motifs for a number of proteases including MMP-11 (15), human kallikrein 2 (35), rat mast cell protease 4 (16), and outer membrane protein T (17). Motifs derived from such an analysis have been shown to be physiologically relevant and used to determine preferred as well as possible alternate protease substrates. The peptides identified have also found utility as substrates suitable for kinetic analysis of protease activity.

In this report we, 1) describe the screening of a 10^12 random 13-mer peptide phage library, resulting in the determination of a 7-amino acid cleavage motif for ADAMTS-4; 2) demonstrate that this motif is specific for ADAMTS-4 and peptides based on this motif are poor substrates for ADAMTS-5; 3) confirm the importance of the P1 glutamic acid in catalysis and substrate recognition; 4) describe the development of a novel fluorogenic peptide substrate and kinetic assay amenable to high throughput screening of ADAMTS-4 inhibitors; and 5) report the results of a BLAST search of the cleavage motif, revealing matrilin-3 as a substrate of ADAMTS-4.

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Peptide Phage Display—A substrate phage library containing 10^12 polypeptide sequences displaying 13 amino acids of randomized sequence equimolar at each position for all amino acids except cysteine was constructed at Dyax Corp. (Cambridge, MA) (36). N-terminal to each displayed randomized sequence was a substrate for ADAMTS-4, these streptavidin-binding epitopes would be cleaved from the phage particle. Non-substrate phage clones that retained the streptavidin-binding epitopes would then be captured by streptavidin-coated surfaces. Initially the library was buffer exchanged in Reaction Buffer by incubating a total of 1.1×10^12 phage in 500 μl of TBS (50 mM Tris, 150 mM NaCl, pH 7.4) plus 1×Roche Complete protease inhibitors minus EDTA (Roche) with 2 μg of streptavidin-coated magnetic beads (Dynal M280, Invitrogen) for 1 h at room temperature in a total volume of 200 μl. The beads were washed 7 times with TBST (TBS, 10% Triton X-100, 10% Tween) and once with TNT (5 mM Tris-HCl, 50 mM NaCl, 10% Triton X-100, pH 7.5). Phage were then eluted from the beads with 200 μl of 15 μM biotin in TNT for 1 h at room temperature, followed by precipitation with 5 μl of 20% PEG8000, 3.5 μM NH4OAc for 1 h on ice. The phage were then resuspended with 500 μl of Reaction Buffer containing 1×protease inhibitors and incubated with 1.1 mM ADAMTS-4 for 1 h at room temperature. Uncleaved phage were removed using 5 μg of streptavidin-coated magnetic beads. Cleaved phage released into the supernatant, and not captured by the beads, were amplified and purified to provide 10^12 phage for further processing through four rounds of selection.

Screening was performed using 96-well plates coated with streptavidin (Pierce) and blocked with 3% bovine serum albumin in phosphate-buffered saline. Individual phage clones were incubated with ADAMTS-4 in Reaction Buffer for 1 h at 37 °C. Treated and untreated phage were then transferred to streptavidin plates and incubated for 1 h at room temperature, washed 5 times with PBST (phosphate-buffered saline, 10% Tween), and bound phage were assayed by incubation with an anti-phage antibody (anti-M13-horseradish peroxidase, GE Healthcare) and developed with TMB peroxidase substrate (KPL, Gaithersburg, MD).

Peptide Sequence Analysis and Motif Generation—A multiple alignment of the peptide sequences was produced with the ClustalW program (20) using the BLOSUM-30 matrix (21). The Pratt motif discovery program was used to identify the conserved patterns in the aligned epitope sequences (22). Peptide sequence logo figures (23) were generated by the local implementation of the WebLogo package (24).

Assay of Proteolytic Activity—Peptides and peptide arrays were either synthesized in house or purchased from New England Peptide (Waltham, MA), or Jerini AG (Berlin, Germany) and were not less than 95% pure. Proteolysis and identification of the scissile bond of substrate peptides was determined by incubating a 10 μM solution of the peptide with 400 pm ADAMTS-4 or ADAMTS-5 for 18 h at 37 °C. 5 μl of each digest was analyzed by reverse phase high performance liquid chromatography (RP-HPLC) on a 75 μm × 150-mm Pepmap column (LC Packings, San Francisco, CA) coupled to a quadrupole.
time-of-flight mass spectrometer (Micromass, Beverly, MA). No prior knowledge of peptide mass was needed. Spectra were acquired in MS mode and MS/MS mode at 1 s/scan. Where MS was not required, 90 μl of each digest was analyzed by RP-HPLC on a 4.6 × 150-mm Vydac C-18 column (The Separations Group, Hesperia, CA) with detection at 214 nm.

Direct kinetic fluorescent analysis of peptide turnover was determined using 6-Fam/QSY-9 (6-carboxyfluorescein, QSY-9-maleimide, Molecular Probes, Eugene, OR) fluorescently quenched peptides detailed in Tables 3 and 4. Briefly, a 1 μM solution of peptide in MMP buffer was incubated with 400 pm ADAMTS-4 or ADAMTS-5 at 37 °C in a total volume of 100 μl. Fluorescence at 519 nm was monitored over a 60-min period with excitation of 495 nm.

**Proteolysis of Matrilin-3 by ADAMTS-4**—Recombinant human matrilin-3 was purchased from R&D Systems (Minneapolis, MN) and reconstituted in MMP buffer at a concentration of 0.5 μg/ml. One μg of matrilin-3 (750 nm) was incubated with 1.5 nM ADAMTS-4 in a total volume of 25 μl at 37 °C. N-terminal protein sequencing was performed by automated Edman degradation on an Applied Biosystems model 494 Pico sequence sequencer. The digests were fractionated by SDS-PAGE (NuPAGE), transferred onto a polyvinylidene difluoride membrane, and stained with Coomassie Blue. The bands of interest were excised and placed into the blot cartridge of the sequencer and the samples were run using blot cycles. Model 610A version 2.1 software was employed for data acquisition and processing.

**ADAMTS-4 Cleavage Motif**—Peptides identified from phage library are specific for ADAMTS-4 and amenable to kinetic analysis of activity. All 50 peptides identified from the peptide phage screen were synthesized and analyzed for ADAMTS-4-mediated cleavage by RP-HPLC. Two peptides that displayed the greatest turnover were peptides B05 (HNEFRQRETYMVF) and B06 (DVQEFRGVTAVIR) (Table 1). LC/MS/MS analysis of peptide B06 was carried out following digestion with ADAMTS-4. Fig. 1 shows the results for peptide B06 with two peptide peaks being identified. After ADAMTS-4 cleavage, one peak with a retention time of 12.151 min was identified as non-cleaved B06, whereas the peak eluting at 11.158 min was identified as the C-terminal fragment of B06 arising from cleavage at Glu/Phe (Fig. 1). The N-terminal fragment was not resolved. Incubation of this same peptide with ADAMTS-5 failed to produce a cleaved product, and only full-length B06 was detected by LC/MS/MS (data not shown). Similarly, no cleavage products were detected for the remaining 48 peptides when assayed with ADAMTS-5, as determined by RP-HPLC. Of the 50 peptides identified from the phage screen, only a subset of the synthesized peptides were analyzed by LC/MS/MS to determine the scissile bond, namely peptides B05, B06, B07, and A08 as well as a peptide based solely on the identified cleavage motif where X-residues were replaced with Ala (data not shown).

Fluorescently quenched versions of B06 and B05 were synthesized using 6-Fam and QSY-9. To increase the solubility of each peptide, the N- and C-terminals were not capped, and amino acids KGK were added to the C terminus, K(6-Fam)-DVQEFRGVTAHIC(Qsy-9)-KGK (Table 2, peptide 6) and K(6-Fam)-HNEFRQRETYMVF(Qsy-9)-KGK (Table 3, peptide 6), respectively. Both peptides were found to give a maximal rate of fluorescence increase at a concentration of 1 μM with an ADAMTS-4 concentration of ~400 pM. Peptide concentrations above 1 μM exhibited an inner filter effect, and the fluorescent yield dropped proportionately with increasing peptide concentration. Following a 10-min equilibration of peptide
B06 to allow the sample to warm to 37 °C, a linear increase in fluorescence was detected for the first 40 min upon addition of ADAMTS-4 (Fig. 2). A slight increase in substrate conversion was noted from 40 to 60 min, indicated by the slight upward curvature of the activity trace possibly due to enhanced solubility of the peptide over time at this temperature. ADAMTS-5 failed to cleave peptide B06, and therefore did not generate a fluorescent signal above background. Similar data were obtained with peptide B05 for both ADAMTS-4 and ADAMTS-5 (data not shown).

$K_m$ values for the 6-Fam/QSY-9 derivatives of B05 and B06 could not readily be determined. Although these peptides were significantly more soluble than the non-fluorescent versions because of the addition of the KGK tails, solubility above 100 $\mu$M was not achievable, and thus meaningful substrate curves could not be generated. $K_m$ values could be approximated using the non-fluorescent versions in competition with the 6-Fam/QSY-9 derivatives. The $IC_{50}$ of the “cold” peptides, Ac-NEFRQRETYMVF-NH$_2$ (Table 3, peptide 1) for B05, and Ac-DVQEFRGVTAVIR-NH$_2$ (Table 2, peptide 1) for B06, were calculated to be $\sim$35 $\mu$M (Fig. 3). As to whether this value represents the true $K_m$ for each peptide, it must be stressed that the competing peptides were significantly different in that they lacked the 6-Fam and QSY-9 labels, as well as the C-terminal KGK extension to aid solubility; therefore, the true $K_m$ could be higher.

A 1 $\mu$M solution of fluorescently quenched peptide B06 was incubated with 400 pM ADAMTS-4 for 1 h at 37 °C. Approximately 31% of the peptide was hydrolyzed in this 1-h period, yielding a velocity of 310 nM/h. The turnover rate of ADAMTS-4 for this peptide substrate was calculated to be 0.22 s$^{-1}$. Because the peptide concentration was significantly less than the estimated $K_m$ for this peptide, $k_{cat}/K_m$ could be calculated directly and was found to be 215,000 M$^{-1}$ s$^{-1}$. Similar analysis of fluorescently quenched peptide B05 yielded a $k_{cat}/K_m$ value of 160,000 M$^{-1}$ s$^{-1}$ for ADAMTS-4 (data not shown).

**Substitution of P1 and P1′ with D-Isomeric Amino Acids**—To gain a better understanding of peptide binding/catalysis by ADAMTS-4, the stereochemistry of P1 and P1′ of peptide B06 was modified. Three peptides were synthesized, containing dGlu at P1 (Table 2, peptide 3), dPhe at P1′ (Table 2, peptide 4), or dGlu at P1 with dPhe at P1′ (Table 2, peptide 5). These peptides were assayed in competition with the 6-Fam/QSY-9
### TABLE 1

Multiple sequence alignment of the ADAMTS-4 epitope sequences with corresponding average percentage of phagemid cleavage and the derived ADAMTS-4 cleavage motif

Predominant amino acids found at a frequency of greater than 40% in a particular position are illustrated with a black background, whereas related amino acids are shown with a grey background.

| Phage Code | Peptide Sequence | Avg. % Cleaved |
|------------|-----------------|----------------|
| D04        | -----MME-RQG-RERVLT | 99.7           |
| B05        | -----HNEV-RQG-AYMP-- | 99.6           |
| B07        | -----NWQIEQ-AKRSAY- | 99.5           |
| D03        | -----LIEK-SN-SVIMWF | 99.4           |
| C02        | -----DYMEVR-RQKQ-WM | 99.4           |
| F03        | -----AELM-RAD-EYHF- | 99.3           |
| D01        | VEHLMR-VKRK-TL-     | 99.2           |
| C05        | -----GVEV-RRQL-PHYM | 99.1           |
| B08        | -----QEIVCAGIETYML-- | 99.0           |
| C06        | -----QEMVRSYV-QKRW- | 98.9           |
| F05        | -----LQSE-RKAP-VDIMW | 98.9           |
| H04        | -----QEEL-RGK1QQPFK | 98.9           |
| E03        | -----QOEMGQGYPWIF    | 98.8           |
| D07        | -----SMETA-ATVISTFE- | 98.7           |
| C07        | -----EQQL-RGQCHIII- | 98.5           |
| B02        | -----MEL-SSQ-RTMYII | 98.5           |
| F01        | -----GAYV-GRWTVYDA | 98.3           |
| B01        | -----QPFMSTPKHTHK-- | 98.2           |
| B06        | -----DVQER-RKV-TAVIR | 98.0           |
| A05        | -----HFA-RTVSHTYLM-- | 97.9           |
| C04        | -----YMEF-RGSTTWFFN- | 97.9           |
| F06        | -----QEMCSY-SMPTN-- | 97.9           |
| E01        | -----HYYMEE-PRDLEM-- | 97.9           |
| A06        | -----NPEAHSSGTVMLR- | 97.8           |
| H01        | -----DHPMEE-RIKTVK-- | 97.8           |
| A01        | -----TPAEM-RCYTVMAL-- | 97.7           |
| F04        | -----GVMVEKGR-RTSIL | 97.7           |
| E03        | -----FQDEFSTG--DIDMDP | 97.6           |
| A04        | -----FQAVEAS-LTHW- | 97.6           |
| D02        | -----YLEDGSTY-RTVWF- | 97.5           |
| F08        | -----TDYLEA-RSQQTHY-- | 97.2           |
| B03        | -----TFQEM-RAPNTSW- | 97.1           |
| B04        | -----FQDVCQIAVYW-- | 96.9           |
| F10        | -----AEM-FAS-LTHYVL | 96.8           |
| D09        | -----DYMEVGSNKISRL-- | 96.7           |
| F09        | -----VIMWEK-GRKTLQ-- | 96.5           |
| D10        | -----FQAER-AKV-ASS- | 96.5           |
| F07        | -----EDYVY-EDVCTTN- | 96.4           |
| A08        | -----QEM-RAHHEAYKLMS- | 96.2           |
| C03        | -----YNEM-RATPEAVV-- | 95.8           |
| H03        | -----PMYHANTRTIVQS- | 95.4           |
| G02        | -----ALEP-SRFLDTIN- | 95.3           |
| C01        | -----WETVAAAP-LMTTWV | 94.3           |
| E02        | -----FQEM-RAAP-TWYY | 94.1           |
| F02        | -----NTLYEV-APPVFYYV- | 90.4           |
| G01        | -----FQPYECDGKTTLM-- | 89.9           |
| A02        | -----KPM-ESRT-FHY- | 88.5           |
| G05        | -----MEI-EGALQRLQRP-- | 82.9           |
| H02        | -----PCQV-RQARKTIE- | 79.4           |
| A07        | -----YRQDDRKHQIHIQY-- | 34.2           |

E-{AFVLMY}-X(0,1){-RK(2,3){-[ST]}-[VYIFWMLA]}
fluorescently quenched peptide B06 (Table 2, peptide 6). Interestingly, the dGlu-modified peptide (Table 2, peptide 3) was able to compete and inhibit cleavage of the fluorescent peptide substrate, displaying an IC50 of \( \frac{1}{10} \) M (Fig. 4). The modified peptide containing dPhe at P1’ (Table 2, peptide 4) was shown to be unfavorable, in that competition with the fluorescent substrate was not detected, indicating that the peptide does not bind the active site of ADAMTS-4. Similarly, substitution of P1 and P1’ with dGlu and dPhe, respectively (Table 2, peptide 5), was also unfavorable, in that this peptide was unable to compete and inhibit cleavage of the fluorescent substrate.

TABLE 2
Synthetic peptides based on peptide B06 identified from the screen

1. Ac-DVQEFRGVTAVIR-NH2
2. Ac-DVQDFRGVTAVIR-NH2
3. Ac-DVQ(e)FRGVTAVIR-NH2
4. Ac-DVQE(f)RGVTAVIR-NH2
5. Ac-DVQ(e)(f)RGVTAVIR-NH2
6. K(6-Fam)-DVQEFRGVTAVIRC(Qsy-9)-KGK

TABLE 3
Synthetic peptides based on peptide B05 identified from the screen

1. Ac-HNEFRQRTMYMVF-NH2
2. KHNEFRQRTMYMVFKGK
3. KHNEFRQRTMYMVFKGK
4. KHNEFRQRTMYMVFKGK
5. KHNEFRQRTMYMVFKGK
6. K(6-Fam)-HNEFRQRTMYVFC(Qsy-9)-KGK

FIGURE 2. Kinetic analysis of ADAMTS-4 activity using a fluorescently quenched peptide substrate. A 1 \( \mu \)M solution of K(6-Fam)-DVQEFRGVTAVIRC(Qsy-9)-KGK was incubated with 400 pM ADAMTS-4 (black dotted line), 400 pM ADAMTS-5 (red dotted line), or by itself (blue dotted line) at 37 °C. Fluorescence was measured at 519 nm, with an excitation of 495 nm.

FIGURE 3. IC50 curves for cold peptides. Peptides B06, Ac-DVQEFRGVTAVIR-NH2 (a), and B05, Ac-HNEFRQRTMYMVF-NH2 (b), were assayed in competition with K(6-Fam)-DVQEFRGVTAVIRC(Qsy-9)-KGK and K(6-Fam)-HNEFRQRTMYMVFC(Qsy-9)-KGK, respectively, at 1 \( \mu \)M and 400 pM ADAMTS-4. Activity as measured as velocity/s (change in RFU/s) is plotted against peptide concentration. RFU, relative fluorescent unit.

To confirm that the dGlu containing peptide is a competitive, non-cleavable peptide inhibitor of ADAMTS-4, reverse phase chromatographic analysis of peptide Ac-DVQ(e)FRGVTAVIR-NH2 was performed (Table 2, peptide 3). Following incubation with ADAMTS-4 for 18 h, no cleavage of the scissile bond was detected, demonstrating that the orientation of the Glu at P1 is critical for catalysis (data not shown). Similar results were obtained for the P1 dGlu variant of peptide B05 (Table 3, peptide 4); the \( \alpha \)-isomeric peptide was able to compete for substrate binding with an IC50 of 8 \( \mu \)M, but was not cleaved by ADAMTS-4 (data not shown). Like the B06 variant, the dPhe and dGlu/dPhe variants of B05 failed to compete and were not cleaved by ADAMTS-4 (data not shown).

To determine the role of P1 Glu in binding and catalysis, P1 was substituted with aspartic acid in peptides B05, KHNDFRQRETYMVFKGK (Table 3, peptide 3), and B06, Ac-DVQDFRGVTAVIR-NH2 (Table 2, peptide 4). Although Asp is a conservative substitution, competition was not observed for either peptide, even at concentrations above 100 \( \mu \)M (Fig. 5), demonstrating that Glu at P1 is needed for cleavage and recognition by ADAMTS-4. To verify that the lack of competition observed in
the kinetic assay was real and not due to preferential cleavage of the 6-Fam/QSY-9-modified peptides by ADAMTS-4 (due to the presence of the 6-Fam and QSY-9 groups that may lower the $K_m$), a competition assay was performed with non-fluorescently quenched native peptide B06 (Table 2, peptide 1) and P1 Asp-substituted B06 (Table 2, peptide 2). Cleavage was then monitored by LC. Similar to the fluorescent kinetic data, little or no inhibition of cleavage of the native peptide was observed, even at concentrations above 100 μM of the P1 Asp competing peptide (data not shown). Longer incubation (24 h) of the P1 Asp-substituted peptide B06 with ADAMTS-4 failed to produce proteolytic products as determined by LC (data not shown). Collectively, the data suggest that Glu at P1 is critical for recognition and cleavage.

**Matrilin-3 Is a Substrate of ADAMTS-4**—BLAST results for the general ADAMTS-4 cleavage motif, E-(AFVLMY)-(0,1)-(RK)-(X2,3)-(ST), produced over 9056 hits in 5742 protein sequences. The proteoglycans versican, brevican, and aggrecan were matches, but interestingly not all of the scissile bonds in aggrecan were represented. Other putative matrix molecules and corresponding scissile bonds are listed in Table 4 and include chondroadherin, cartilage intermediate protein I, and matrils 1–4, all cartilage components. Two potential sites of cleavage within matrilin-3 were predicted from the BLAST results for the ADAMTS-4 cleavage motif, Glu98/Phe99 and Glu435/Ala436 (Fig. 6). We were interested in determining if matrilin-3 could possibly be an ADAMTS-4 substrate.

Incubation of ADAMTS-4 with recombinant human matrilin-3 (50 kDa) produced three bands after 30 min at 37 °C, as visualized by SDS-PAGE (Fig. 7). N-terminal sequencing of each band identified the 50-kDa species as mature, intact, non-proteolyzed matrilin-3; the 45-kDa species (with the native N-terminal of matrilin-3) results from truncation at the C terminus, and a 5-kDa protein bearing the N-terminal sequence, 436ARRLVS, corresponds to cleavage of matrilin-3 at one of the predicted sites of cleavage at Glu98/Ala436. Interestingly, no other proteolytic fragments were detected even though the BLAST results identified a potential site of cleavage in the von Willebrand factor A (VWF A) domain of matrilin-3 at Glu98/Phe99. To verify that the proteolysis of matrilin-3 was ADAMTS-4 mediated and not due to a contaminating protease, the small molecule aggrecanase inhibitor, SC81956, was included in the digest and no cleavage of matrilin-3 was observed after 24 h (Fig. 7, lane 9). Additionally, recombinant ADAMTS-5, expressed and purified in an analogous manner as ADAMTS-4, failed to process matrilin-3, producing no detectable proteolytic fragments (data not shown).

**DISCUSSION**

ADAMTS-4 has been shown to play a major role in the initiation and progression of osteoarthritis, in human cartilage, through the proteolysis of aggrecan as well as other proteoglycan and non-proteoglycan substrates, predominantly at sites containing a Glu at the P1 residue of the scissile bond. Alignment of the known ADAMTS-4 cleavage sites has failed to produce a concise motif unique to this protease. We report for the first time the derivation of a 7-amino acid cleavage motif specific for ADAMTS-4 relative to ADAMTS-5: E-(AFVLMY)-(0,1)-(RK)-(X2,3)-(ST)-(VYIFWMLA). Surprisingly, ADAMTS-5 failed to cleave synthetic peptides based on the ADAMTS-4 cleavage motif even though ADAMTS-4 and ADAMTS-5 share identical sites of cleavage in aggrecan (Fig. 2 and data not shown).

It has been shown that deglycosylation of aggrecan or removal of the thrombospondin domain of ADAMTS-4 impedes proteolysis, indicating that exosite interactions are a strong component of ADAMTS-4-mediated proteolysis of aggrecan as well (25). A question that arises from the data is why are the peptides identified from the phage screen substrates of ADAMTS-4, given that this motif is extremely similar to the scissile bonds in aggrecan, yet aggrecan is not cleaved when the GAG chains are removed? The answer could lie in substrate presentation. The peptide substrates are short 13-mers, likely lacking any rigid secondary structure and could conceivably conform to the active site of ADAMTS-4, contrasting with a protein substrate where the scissile bond is likely held in a more rigid three-dimensional conformation. A $K_m$ value for the peptide was not determined due to solubility issues, but a lower limit was estimated to be $\sim 35$ μM, representing modest affinity. Conformational restraint of the scissile bond coupled with a mediocre affinity for the core protein may then explain why deglycosylated aggrecan is no longer a substrate of ADAMTS-4.

Although the $K_m$ for the synthetic peptides was average, they did prove valuable as fluorescently quenched substrates for the kinetic analysis of ADAMTS-4 activity (Fig. 2). Solubility of both peptides (B05 and B06)
was enhanced by the inclusion of a Lys at the N-terminal, and Lys-Gly-Lys at the C-terminal of each peptide. Solubility was further enhanced by the addition of the long-range quencher QSY-9 (Molecular Probes). Although a $K_m$ could not be measured directly, assay of ADAMTS-4 activity at a concentration of 1 $\mu$M 6-FAM/QSY-9 peptide B05 or B06, which is significantly lower than the $K_m$ (35 $\mu$M), allowed for the determination of the specificity constant ($k_{cat}/K_m$) for each peptide. $k_{cat}/K_m$ values for peptides B06 and B05 were calculated to be 215,000 and 160,000 (M$^{-1}$ s$^{-1}$), respectively, and are in agreement with values published for coumarin-labeled PUMP and gelatinase peptide substrates, where $k_{cat}/K_m$ ranged from 169,000 to 690,000 (M$^{-1}$ s$^{-1}$), respectively (26), but are an order of magnitude larger than MMP-11 peptide substrates identified by phage display (15), or coumarin-labeled stromelysin 1 peptide substrates (27). Although the fluorescently quenched peptides B05 and B06 are amenable to the high throughput screening of ADAMTS-4 inhibitors, it should be stressed that molecules that disrupt positive exosite interactions between ADAMTS-4 and a natural substrate may not be identified using these short cleavage motif peptides as substrates.

The importance of the P1 Glu and P1’ Phe residue was examined in both peptides B05 and B06, either by changing the stereochemistry of the Glu or Phe residues, or by substituting P1 Glu with Asp. Interestingly, substitution with dGlu at P1 in both peptides was favorable, whereas inclusion of dPhe was not tolerated (Fig. 4). The dGlu substitution seemed to enhance binding in that the measured IC50 for peptides B05 and B06 with P1 dGlu was $\approx$8–10 $\mu$M compared with 35 $\mu$M for the native sequences. It can be hypothesized that substitution at P1 with dGlu produces a favorable kink in the peptide chain, allowing for enhanced substrate binding; future homology modeling will address this. Although the dGlu-substituted peptides bound ADAMTS-4, they were not cleaved, suggesting that orientation of the Glu residue is critical for catalysis. Perhaps this is mediated through specific interaction of the Glu residue with the amino acid side chains in the catalytic cleft of ADAMTS-4. In addition, inclusion of dGlu may indirectly disrupt scission of the peptide by displacing favorable P and P’ interactions, resulting in misalignment of the P1 residue. Substitution at P1’ with dPhe, on the other hand, produced peptides that were not recognized or cleaved by ADAMTS-4, implying P1’ binding into the S1’ pocket of ADAMTS-4 is very stringent with regard to stereochemistry. Examination of Table 1 clearly shows the importance of P1 being occupied by glutamic acid. Conservaive replacement of P1 with Asp in peptides B05 and B06 failed to produce a peptide that would compete for binding with the native P1 Glu-substituted peptides (Fig. 5).

So, if P1 Glu is critical for binding and catalysis, how does ADAMTS-4 cleave $\alpha_2$M or undergo autocatalytic processing where P1 is not Glu but Met or [Lys, Thr], respectively? An examination of the 50 peptides identified from the phage screen also reveals the presence of peptide substrates devoid of a Glu residue, $\approx$16% (Table 1). Peptide D04 (Table 1) resembles the cleavage site in $\alpha_2$M, MFKG versus MGRG, respectively, where M is P1.

For $\alpha_2$M and autocatalytic processing, the contribution of substrate presentation may be important, even though the cleavage motifs in these proteins may not be optimal compared with the peptide derived motif that we have identified. Further evidence weakens the contribution of amino acids upstream from P1 with respect to substrate recognition, based on preliminary data that show that N-terminal truncation up to the P1 Glu of peptide EFRQRETYMVFKGK (Table 3, peptide 5) is not detrimental to binding/recognition (data not shown). Collectively, these data suggest that P1’ amino acids play a predominant role in substrate recognition, binding, and catalysis. More studies are required to address this subject.

Results from the BLAST search using the motif E-(AFV-LMY)-X(0,1)-(RK)-X(2,3)-(ST) produced over 9000 hits in $\approx$5700 proteins. Apart from aggrecan and the proteoglycans versican and brevican, other cartilage proteins were identified including: cartilage homeoprotein 1, cartilage intermediate layer protein-1 and -2 precursors, and matrilins 1–4. The matrilin family is highly conserved and ubiquitously expressed, including: cartilage homeoprotein 1, cartilage intermediate layer protein-1 and -2 percursors, and matrilins 1–4. The matrilin family is highly conserved and ubiquitously expressed, including: cartilage homeoprotein 1, cartilage intermediate layer protein-1 and -2 percursors, and matrilins 1–4. The matrilin family is highly conserved and ubiquitously expressed, including: cartilage homeoprotein 1, cartilage intermediate layer protein-1 and -2 percursors, and matrilins 1–4. The matrilin family is highly conserved and ubiquitously expressed, including: cartilage homeoprotein 1, cartilage intermediate layer protein-1 and -2 percursors, and matrilins 1–4.
cleavage of matrilin-3 at Glu\(^{335}/\text{Ala}^{336}\). Interestingly, cleavage within the VWF A domain was not detected, even though this site matched the cleavage motif for ADAMTS-4. This site may be cryptic in the intact matrilin-3 molecule, and therefore unavailable for proteolysis, whereas in the matrix this site may be exposed due to the propensity of matrilin-3 to form interactions with other matrix components. The list of ADAMTS-4 substrates continues to grow and now includes matrilin-3. The fact that ADAMTS-5 failed to cleave matrilin-3 suggests that ADAMTS-4 may represent a more matrix destabilizing force in OA. To date ADAMTS-4, but not -5, has been shown to cleave multiple cartilage matrix proteins other than aggregan, including cartilage oligomeric matrix protein (12), biglycan (32), and now matrilin-3.

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