Identification of Peptide Substrates for Human MMP-11 (Stromelysin-3) Using Phage Display

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The MMP-11 proteinase, also known as stromelysin-3, probably plays an important role in human cancer because MMP-11 is frequently overexpressed in human tumors and MMP-11 levels affect tumorogenesis in mice. Unlike other MMPs, however, human MMP-11 does not cleave extracellular matrix proteins, such as collagen, laminin, fibronectin, and elastin. To help identify physiologic MMP-11 substrates, a phage display library was used to find peptide substrates for MMP-11. One class of peptides containing 26 members had the consensus sequence A(A/Q)(N/A)↓(L/Y)(T/V/M/R)(R/K), where ↓ denotes the cleavage site. This consensus sequence was similar to that for other MMPs, which also cleave peptides containing Ala in position 3, Ala in position 1, and Leu/Tyr in position 1′, but differed from most other MMP substrates in that proline was rarely found in position 3 and Asn was frequently found in position 1. A second class of peptides containing four members had the consensus sequence G(G/A)E↓LR. Although other MMPs also cleave peptides with these residues, other MMPs prefer proline at position 3 in this sequence. In vitro assays with MMP-11 and representative peptides from both classes yielded modest $k_{cat}/K_m$ values relative to values found for other MMPs with their preferred peptide substrates. These reactions also showed that peptides with proline in position 3 were poor substrates for MMP-11. A structural basis for the lower $k_{cat}/K_m$ values of human MMP-11, relative to other MMPs, and poor cleavage of position 3 proline substrates by MMP-11 is provided. Taken together, these findings explain why MMP-11 does not cleave most other MMP substrates and predict that MMP-11 has unique substrates that may contribute to human cancer.

MMP-11, also known as stromelysin-3, is one of more than 20 matrix metalloproteinases (MMP) (reviewed in Ref. 1). The MMP-11 gene was originally identified by screening a breast cancer cDNA library for genes that were expressed at higher levels in invasive carcinomas than in breast fibroadenomas (2). Additional work has shown that MMP-11 is usually overexpressed in many human carcinomas, including breast, non-small cell lung, and colorectal carcinomas, but is rarely expressed in normal tissue, including the normal tissue surrounding the tumor (3–5). In fact, adults only express MMP-11 in tumors and regenerating or healing tissues (reviewed in Ref. 6). Furthermore, MMP-11 expression correlated with a shorter recurrence-free survival for breast cancer patients, providing further support for an important role for MMP-11 in breast cancer (5, 7, 8).

Experiments with mice or fibroblasts lacking MMP-11 also suggest that MMP-11 plays an important role in human cancer. First, mice lacking MMP-11 have a reduced incidence and size of tumors induced by 7,12-dimethylbenzanthracene compared with mice with a functional MMP-11 (9). Second, MMP-11 null fibroblasts, unlike wild type fibroblasts, do not stimulate the implantation rate of MCF7 xenografts in nude mice (9). Third, syngeneic tumor cells had a higher rate of apoptosis when injected into MMP-11 null mice compared with wild type mice (10). Hence, the frequent overexpression of MMP-11 in human tumors and the effect of MMP-11 levels on tumor formation in mice both suggest that MMP-11 plays an important role in human cancer.

Relatively little is known about the physiologic substrates for MMP-11. In particular, human MMP-11, unlike most MMPs, does not readily cleave extracellular matrix components, such as collagen, laminin, fibronectin, or elastin (11, 12). In vitro studies showed, however, that MMP-11 cleaves α1-proteinase inhibitor, α2-macroglobulin, and insulin-like growth factor-binding protein-1, although the physiologic significance of these reactions is unknown (11, 13). More importantly, recent experiments showed that MMP-11 mutants without proteolytic activity did not stimulate the implantation of MCF7 cells into nude mice (14). These studies strongly suggest that MMP-11 has proteolytic activity that is important for its role in cancer.

In addition to the poor cleavage of extracellular matrix components, human MMP-11 differs from other MMPs in that it contains alanine at residue 235, whereas mouse MMP-11 and other MMPs contain proline at the corresponding residue (12). This alanine residue affects catalytic activity because the human protein MMP-11-A235P, but not MMP-11, readily cleaved laminin and type IV collagen. In fact, human MMP-11-A235P had activity similar to that of mouse MMP-11. It is unclear whether the alanine 235 to proline mutation in human MMP-11 affects catalytic activity, substrate dependence, or both.

In this study, we used phage display libraries to select peptide substrates for human MMP-11. Preferred sequences for MMP-11 cleavage could identify physiologic substrates for...
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MMP-11. Such substrates would be interesting because they are likely to be important for human cancer.

EXPERIMENTAL PROCEDURES

Construction of Phage Display Library—A phage display library was constructed containing a random hexahistidine peptide sequence expressed between a hexahistidine tag and the mature gene III protein of M13. To construct this library, the following two oligonucleotides were annealed, elongated with the Klenow fragment in the presence of nucleotide triphosphates, and then digested with KpnI and BamHI (first and second underlined segments, respectively): 5′-gctggtcagaattctggtaaaacggtgtagttgctagaaggtggt-3′ and 5′-nccgccgcctaggtgtag-3′, where n indicates equimolar a, t, c and g and s indicates equimolar c and g. The digested DNA fragment was then ligated with the vector M13Pl9, which was similarly digested with KpnI and BamHI, and the ligation mixture was transformed into Escherichia coli strain K91 (thi1/HisR) yielding 4.4 × 10^10 independent transformants. The resulting phage mixture was amplified in K91 cells and concentrated by polyethylene glycerol precipitation to yield 4.7 × 10^13 plaque-forming units/ml. The resulting phage expressed a protein containing: gene III leader sequence-Ala-His_Gly_Xaa_Gly_Gxx-3′ gene III mature protein.

Construction of Control Phage Clones—Positive and negative control phage were constructed to help develop methods for library screening. A positive control phage was constructed with oligonucleotides that expressed the hexapeptide PLG ↓ LYA because this sequence is known to be a substrate for MMP-14. Two clones containing the hexapeptides MTIQMIS or TALSPQ were isolated from the library that were not cleaved by MMP-14.

Expression Vectors for MMP-14 and MMP-11—Fragments of human MMP-14 and MMP-11 that encoded the catalytic domains were inserted into vectors for expression in E. coli. For MMP-11, a DNA fragment encoding residues 98–272 was amplified by PCR from IMAGE clone 2425546 (Research Genetics, Birmingham, AL) with a 5′ oligonucleotide that contained NdeI and a 3′ oligonucleotide that contained BglII. The DNA fragment was digested with NdeI and BglII and then ligated with pET3b (Novagen, Madison, WI) that was digested with NdeI and BgIII. MMP-11 mutants with increased activity, MMP-11-A235P, or decreased activity, MMP-11-E216A, were generated by site-directed mutagenesis. DNA sequencing confirmed the authenticity of the preceding plasmids.

For MMP-14, a vector that directed the expression of MMP-11 residues 111–208 was created as previously described (15).

Purification of MMP-14 and MMP-11 Catalytic Protein Fragments—Plasmids that expressed the catalytic regions of MMP-11 or MMP-14 were transformed into BL21pLysS (Novagen). Recombinant proteins were induced and purified based on published methods (15). Briefly, inclusion bodies were purified from E. coli and were induced to express the desired recombinant protein for 3 h at 37 °C. The purified inclusion bodies were dissolved in buffer with 8 M urea, dialyzed, and dialyzed against buffer with decreasing amounts of urea to facilitate refolding. Insoluble protein was then removed by centrifugation, and the resulting proteins were stored at –80 °C. The identity of the MMP-11 was confirmed by Western blotting with antibody SL305 (Oncogene Sci.). The concentration of the soluble protein was determined by comparison with bovine serum albumin following Coomassie Blue staining of SDS-polyacrylamide gels.

Screening the Phage Display Library with MMP-14, MMP-11, or MMP-11-A235P—0.1 ml of phosphate-buffered saline containing ~10° phage was added to 96-well Reacti-Bind Metal Chelate Plate (Pierce), and the resulting plate was shaken gently overnight at room temperature. Unbound phage were removed by washing six times with 0.2 ml of phosphate-buffered saline containing 0.05% Tween 20. This procedure yielded ~2–5% of the input phage bound to the plate based on titering phage that were eluted from the plate with 1 ml of 0.2% EDTA for 10 min. To enrich for MMP-14 substrates, the bound phage were digested in 0.1 ml of 50 mM Hepes, 10 mM CaCl_2, 100 mM NaCl, 0.1% Brij 30, 10 ng MMP-14 (ph 7.5) for 6 h at 37 °C with gentle shaking. The resulting supernatants were then collected and amplified, and the enrichment cycle was repeated as above. Individual phage were cloned and analyzed as described under “Results.” Likewise, bound phage were digested in 0.1 ml of 50 mM Tris-Cl, 10 mM CaCl_2, 100 mM NaCl (ph 7.5) with either 500 nM MMP-11 or 180 nM MMP-11-A235P for 6 h at 37 °C with gentle shaking to enrich for MMP-11 and MMP-11-A235P substrates. The resulting supernatants were then collected and amplified, and the enrichment cycle was repeated three more times. Individual phage were cloned, amplified, and analyzed as described under “Results.” In some cases, the amount of phage released from the nickel plate was determined by titration of the supernatant and/or phage eluted from the plate after a 10-min treatment with 0.1 ml of 0.02 M EDTA.

Enzyme Assays with Peptides—2 ml of recombinant enzyme were dialyzed twice against 1 liter of 50 mM Hepes, 100 mM NaCl, 5 mM CaCl_2, 1 μM ZnCl_2, 5 mM β-mercaptoethanol (ph 7.6) (reaction buffer plus β-mercaptoethanol) to remove amines that would react with fluororescine. Peptides (Research Genetics, Huntsville, AL) were dissolved in Me_2SO at 10 mM. The peptide at concentrations ranging from 0.016 to 1 mM was diluted into reaction buffer for a final volume of 0.1 ml and equilibrated to 37 °C. Enzyme at concentrations from 1 to 65 nM was then added, and the reaction was continued for 1–20 h. To quantify the extent of hydrolysis, 0.05 ml of 1 mg/ml fluorescamine in acetonitrile/MeOH 1:1 was added followed by 0.05 ml of 1 mg/ml fluorescamine in acetonitrile and gentle mixing. The fluororesceral signal generated by the reaction of the fluorescamine with the primary amines created by enzymatic digestion of the peptides was measured using a plate reader with excitation and emission wavelengths of 390 and 490 nm, respectively. The molar amount of fluorescamine-peptide conjugate was determined using standards prepared from fluorescamine. These values were then used to calculate the amount of product formed (16). k_cat/K_m was calculated from nonlinearity regression analysis of Michaelis-Menten plots using Enzyme Kinetics software (Trinity Software).

As indicated under “Results,” the digestion of some peptides was also monitored using reversed-phase HPLC separation. In these cases, the reaction was stopped with 10 mM EDTA, and the reaction products were separated using a C18 reversed-phase HPLC column and acetonitrile gradient. The peptide fragments were detected by their absorbance at 210 nm. The results obtained by the HPLC method were in excellent agreement with the results obtained with the fluorescamine method. Finally, analysis of the digestion of peptides MA15 and MA18 with either the wild type or the A235P mutant MMP11 was performed using a Waters Micromass ZQ mass spectrometer interfaced with a Shimadzu HPLC. Analysis was performed using either acetonitrile, water, 0.1 mM ammonium acetate or methanol, water, 0.1% trifluoroacetic acid gradients as mobile phase on a C18 reversed-phase HPLC column using electrospray ionization (+) ionization.

Gel-based Activity Assay for MMP-11 Cleavage of a-Proteinase Inhibitor—MMP-11 α-antitrypsin inhibitor cleavage activity was measured by incubating 5 μg of α-proteinase inhibitor (Calbiochem 178251) in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM CaCl_2, 1 μM ZnCl_2, 26 mM MMP-11 or MMP-11-A235P in a total volume of 0.65 ml at 37 °C for the indicated times. The reactions were stopped by adding 10 μl of 3 mM β-mercaptoethanol, 10% SDS, 50 mM Tris-HCl (pH 7.6), 25% glycerol. The reaction products (15 μl) were separated using an 18% Tris-HCl gel (Bio-Rad) and visualized with Coomassie Blue.

RESULTS

Identification of MMP-14 Substrates Using Phage Display—A phage display library was constructed to identify peptide substrates for proteases. In this system, each phage expresses five copies of a hexahistidine peptide followed by a random hexapeptide on its surface. Proteolytic digestion within the hexahistidine removes the hexahistidine tag, thereby allowing separation of phage where the hexapeptide was cleaved from phage that were uncleaved.

A screen for MMP-14 substrates was used to test this phage display system. A phage clone that contained a hexapeptide that was cleaved by MMP-14, PLG ↓ LYA, and two clones with hexapeptides that were not cleaved by MMP-14, MTIQMIS and TALSPQ, were first used to develop methods for library screening. Based on these studies, conditions were identified where ~75% of the positive control phage were released from the nickel plate, whereas less than 2% of the negative control phage were released from the nickel plate (see “Experimental Procedures” for details.) Using these conditions, two rounds of enrichment were performed with MMP-14. 52 random phage clones from this enriched pool were then individually digested with MMP-14 or buffer controls, and the fraction of the phage released by MMP-14 was determined. This analysis showed that 31 of 52 clones were released from nickel plates at levels equal to or greater than the level for the positive control phage. Some of these release rates are quantified in Table I. DNA from...
these 31 phage were sequenced, and the predicted hexapeptides were aligned (Table I). Based on this alignment, a consensus sequence of PL(G/P/A)$_2$L(R/M) was obtained (Table II). The individual peptide sequences and the consensus sequence were in excellent agreement with preceding identification of MMP-14 substrates using phage display and peptide methods (17–19). Hence, our studies substantiate the conclusions of other investigators and validate our phage display method for identifying peptide substrates.

Identification of MMP-11 Substrates Using Phage Display—A fragment of human MMP-11 containing residues 98–272 was expressed in E. coli. Two MMP-11 mutants were also expressed in E. coli to aid in the identification of MMP-11 substrates. One mutant, MMP-11-E216A, altered a residue required for catalytic function (14), whereas a second mutant, MMP-11-A235P, had increased activity for cleavage of /H9251 $\alpha$-proteinase inhibitor (12) (Fig. 1A). This analysis showed that both MMP-11 and MMP-11-A235P cleaved /H9251 $\alpha$-proteinase inhibitor, yielding the expected products. Furthermore, MMP-11-A235P was about 10-fold more active than MMP-11, consistent with previous results (12). Similar results were obtained using either /H9251 $\beta$-casein, /H9251 $\gamma$-macroglobulin, or insulin-like growth factor-binding protein-1 as a substrate (data not shown). The cleavage of /H9251 $\alpha$-proteinase inhibitor was inhibited by AG3340, a broad spectrum MMP inhibitor (20), with an IC$_{50}$ of ~6 nM (Fig. 1B).

MMP-11 and MMP-11-A235P were then used to enrich the phage display library for substrates. Unfortunately, neither the positive control phage clone for MMP-14 (PLG$_2$LYA) nor any of the phage clones selected with MMP-14 were good substrates for MMP-11 or MMP-11-A235P. In the absence of phage substrates to establish conditions for screening, enrichment conditions were chosen based on the published activity of MMP-11 and MMP-11-A235P (see “Experimental Procedures” for details). Using these conditions, ~25-fold more phage were released from the pool of phage that had undergone four rounds of enrichment than from the nascent phage library. 60 phage from both the MMP-11 and MMP-11-A235P enrichments were then individually cloned and tested for release from nickel plates by MMP-11, MMP11-A235P, or buffer controls. This analysis showed that 7 of 60 phage clones from the MMP-11 enrichment and 34 of 60 phage clones from the MMP-11-A235P enrichment were released from nickel plates at 100-fold or higher levels than the negative control clones. This analysis also showed that all phage released by MMP-11 could be re-

### Table I

| Phage clone | Putative subsite | Hydrolysis |
|-------------|------------------|------------|
| 14-A40      | I P Q G L L (G)  | 77         |
| 14-B4       | L P R G L Q (G)  | 70         |
| 14-B15      | (G) I G L R L   | 64         |
| 14-A21      | Q P M G L F (G) | 65         |
| 14-B21      | K P I G L M (G) | 60         |
| 14-B11      | (G) P L G M M  | 61         |
| 14-A12      | (G) P R G L T | 33         |
| 14-B3       | L A M G M S     | 61         |
| 14-A18      | K P S G L W (G) | ND         |
| 14-B7       | T P S G L W (G) | ND         |
| 14-B32      | (G) P A G L Q (G) | 70         |
| 14-A11      | (G) P L P M I | 67         |
| 14-B17      | (G) P L P L K | 54         |
| 14-A44      | Q P L P M K (G) | 49         |
| 14-A31      | F P L P L L (G) | 58         |
| 14-A7       | F A N P L (G)   | ND         |
| 14-B6       | S G E P L L (G) | ND         |
| 14-B18      | I P V P L W (G) | ND         |
| 14-B20      | Q P Q P L W (G) | ND         |
| 14-B25      | (G) P A P L R E | ND         |
| 14-A41      | I P R A I M (G) | 58         |
| 14-A8       | S P L A L L (G) | 65         |
| 14-A20      | R P Y A M S (G) | 34         |
| 14-B31      | L P Q A L Q (G) | 34         |
| 14-B8       | (G) P L A L T | ND         |
| 14-B13      | Q P A L T (G)   | ND         |
| 14-B19      | (G) P A L V K | ND         |
| 14-B22      | L P L A L I | ND         |
| 14-A15      | N P L S L R (G) | 81         |
| 14-B14      | (G) P A S R L | ND         |
| 14-B16      | H P L S L M (G) | ND         |
| + control   | (G) P A S L R (G) | ND         |
| − control   | (G) T A L S P Q | 2          |
| − control   | (G) M T Q M I S | 2          |

* Phage clones isolated from screens using MMP-14. Predicted peptide sequences were grouped based on their putative position 1 residue.

* Percentage of phage released from nickel plate with 3 nM MMP-14.

* (G) encoded by vector sequences.

* Not determined.

### Table II

| Putative subsite | Hydrolysis (P) |
|------------------|---------------|
| P (90) | L(36) | G(39) | L(86) | R(23) |
| P(32) | A(29) | M(16) | M(16) |

* Residues occurring in more than 15% of 31 peptides in Table I were included in this analysis.
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Fig. 1. Cleavage of α1-proteinase inhibitor by recombinant MMP-11 and MMP-11-A235P. A, α1-proteinase inhibitor was incubated with MMP-11 or MMP-11-A235P for the indicated times. MMP-11 cleaved α1-proteinase inhibitor with a half-time of 1 h, whereas MMP-11-A235P cleaved most of the substrate within 1 and 2 h. Unreacted α1-proteinase inhibitor was loaded in the left lane for comparison. B, α1-proteinase inhibitor was incubated with MMP-11 and the indicated amount of AG3340, an MMP inhibitor for 1 h. AG3340 prevented MMP-11 cleavage of α1-proteinase inhibitor with an approximate IC₅₀ of 6 nM. Unreacted α1-proteinase inhibitor was loaded in the left lane for comparison.

The extent of cleavage was determined using fluorescamine, which reacted with the primary amines created by peptide cleavage to generate fluorescent conjugates. Nonlinear regression analysis of Michaelis-Menten plots was used to determine $k_{cat}$ and $V_{max}$ values that were then used to calculate $K_m$,$k_{cat}/K_m$ values (Table V). Although comparisons between the columns of Table V are subject to the vagaries of the percentage of active enzyme after refolding, certain conclusions are evident. For instance, peptide MA13, which contained the hexapeptide PLG↓LYA used for the positive control phage in the MMP-14 screen, was a good substrate for MMP-14 but was not cleaved at a detectable rate by MMP-11 or MMP-11 mutant proteins, as expected from the phage assays (Table V). MA15 and MA18 contained the hexapeptides GAN↓LVR and YAE↓LRM that represented the two major classes of sequences identified from the MMP-11 phage screens. Consistent with the phage assays, both MA15 and MA18 were good substrates for MMP-11, better substrates for MMP-11-A235P, and not cleaved at detectable rates by either MMP11-E216A or MMP-14 (Table V). MA16 and MA17 contained hexapeptides QPRPGVW and TDAWLVS from the two phage clones isolated in the MMP-11 screen that were not represented by either of the major classes. Surprisingly, neither MA16 nor MA17 were cleaved at detectable rates by MMP-11 or MMP11–235A. Possible reasons for the isolation of these sequences in the phage display screen will be considered under "Discussion." As expected from the sequence, MA16 was a good substrate for MMP-14. The results using the fluorescamine assay with MA16 and MA17 were confirmed by HPLC analysis (data not shown). MA20 contained the hexapeptide PLA↓LWA that was previously found to be a good MMP-14 substrate and a weak substrate for mouse MMP-11 (21). Consistent with these results, MA20 was a good substrate for MMP-14, a weak substrate for MMP-11-A235P, and a poor substrate for MMP-11 (Table V). Finally, MA21 contained the decapetide GAAGA↓MFLEA that spanned the natural MMP-11 cleavage site in α1-proteinase inhibitor. As expected, MA21 was a good substrate for MMP-11, a better substrate for MMP-11-A235P, and not cleaved at detectable rates by MMP-14. Based on these results, we conclude that MMP-11 and MMP-11-A235P cleaved hexapeptides from the two classes identified using MMP-11 at rates that were similar to those for other MMPs with their preferred peptide substrates. Furthermore, MMP-11-A235P cleaved these substrates at rates 2–5-fold faster than MMP-11, with $K_m$ values—2-fold lower than wild type MMP-11 (Table VI). The site of cleavage indicated in Tables V and VI was determined using a combination of HPLC separation of the reaction products after the digestion of peptides MA15 or MA18 with wild type MMP11 or the mutant MMP-11-A235P with subsequent determination of the molecular weight of the product peaks by mass spectroscopy. Fragments consistent with the proposed cleavage sites were observed for both peptides.

Structural Explanations for MMP-11 Enzymatic Properties—X-ray crystallography has shown that the catalytic domains of MMPs have a very similar architecture consisting of a five-stranded β sheet and three α helices (22, 23). Differences between these structures occur mainly at the S1 "specificity loop" and the loop just above it connecting β strand 5 with helix 2. The recent structure of mouse MMP-11 shows that it has most of the common structural features of the MMPs (Fig. 2) (24). These structural comparisons form the basis for an explanation of the increased activity of mouse MMP-11 and human MMP-11-A235P relative to human MMP-11. In particular, human residue Ala²³⁸ corresponds to mouse Pro²³⁸ based on se-
sequence alignments (data not shown). Pro239 of mouse MMP-11 overlays Pro217 of MMP-8 (25), part of the conserved MXP motif that forms a turn directly below the catalytic site (Fig. 2) and is likely to interact with the substrate backbone. Hence, replacement of a turn-inducing proline by alanine is likely to disrupt this loop and, consequently, the interaction of the substrate with the active site.

These structural comparisons also form the basis for an explanation of the poor cleavage by MMP-11 of substrates having proline in position 3. Fig. 2 indicates one major difference between these MMPs is the presence of a leucine residue in position 2 of MMP-11 which is replaced by threonine in MMP-8. Leucine is larger and bulkier than threonine, and this may restrict the movement of the substrate backbone in the active site.

**TABLE III**

Predicted peptide sequences selected by MMP-11 digestion

| Phage clone<sup>a</sup> | Putative subsite |
|-------------------------|------------------|
|                         | P4   | P3   | P2   | P1   | P1<sup>′</sup> | P2<sup>′</sup> | P3<sup>′</sup> | P4<sup>′</sup> |
| Group A                 |      |      |      |      |            |            |            |            |
| 11-19                   | A    | Q    | N    | L    | V    | K           |            |            |            |
| 11-45                   | A    | A    | N    | L    | V    | K           |            |            |            |
| 11-47                   | A    | A    | N    | L    | V    | K           |            |            |            |
| 11-50                   | S    | A    | N    | Y    | T    | M           |            |            |            |
| 11-56                   | A    | Q    | N    | Y    | T    | R           |            |            |            |
| 11AP-6                  | W    | A    | N    | L    | T    | K           |            |            |            |
| 11AP-7                  | A    | A    | N    | L    | V    | R           |            |            |            |
| 11AP-27                 | A    | A    | N    | L    | V    | R           |            |            |            |
| 11AP-15                 | A    | A    | N    | L    | R    |             |            |            |            |
| 11AP-16                 | (G)<sup>b</sup> | A    | N    | Y    | I    | V            | K           |            |            |
| 11AP-28                 | A    | G    | N    | L    | M    | M           |             |            |            |
| 11AP-52                 | (G)  | A    | N    | L    | I    |             | K           |            |            |
| 11AP-B2                 | M    | A    | A    | N    | Y    | V           |             |            |            |
| 11AP-B11                | A    | A    | N    | Y    | M    | M           |             |            |            |
| 11AP-B15                | A    | A    | N    | L    | R    | L           |             |            |            |
| 11AP-B16                | A    | Q    | N    | L    | M    | R           |             |            |            |
| 11AP-B5                 | S    | A    | N    | Y    | I    |             | S           |            |            |
| 11AP-11                 | (G)  | A    | A    | L    | T    | A            | K           |            |            |
| 11AP-40                 | (G)  | A    | A    | L    | R    | M            | Y           |            |            |
| 11AP-60                 | (G)  | A    | A    | Y    | T    | K            | K           |            |            |
| 11AP-B3                 | A    | Q    | A    | L    | R    | I           |             |            |            |
| 11AP-B4                 | (G)  | A    | A    | M    | L    | M            | V           |            |            |
| 11AP-B10                | A    | Q    | A    | Y    | T    | R           |             |            |            |
| 11AP-B12                | A    | S    | A    | L    | R    | M           |             |            |            |
| 11AP-20                 | (G)  | Q    | N    | M    | T    | M            | P           |            |            |
| 11AP-B14                | (G)  | A    | S    | M    | M    | K            | A           |            |            |
| Group B                 |      |      |      |      |            |            |            |            |
| 11AP-35                 | (G)  | (G)  | E    | L    | R    | T            | S           | K           |
| 11AP-39                 | (G)  | A    | E    | L    | R    | Q            | K           |             |
| 11AP-41                 | (G)  | (G)  | E    | L    | R    | L            | A           | P           |
| 11AP-B7                 | Y    | A    | E    | L    | R    | M           |             |            |
| Other sequences         |      |      |      |      |            |            |            |            |
| 11-36                   | Q    | P    | R    | G    | V    | W           |             |            |
| 11-38                   | T    | D    | A    | W    | L    | S           |             |            |

<sup>a</sup> Phage clones selected with MMP-11 or MMP-11A235P are designated as 11-<i>x</i> or 11AP-<i>x</i>, respectively. The sequences are grouped based on their position 1 (P1) residues.

<sup>b</sup> (G) encoded by vector sequences.

**TABLE IV**

Subsite preferences from Group A hexapeptides selected by MMP-11

| Putative subsite | Residue (%)<sup>a</sup> |
|------------------|--------------------------|
|                  | A(58)                    |
|                  | (G)<sup>b</sup> (31)     |
|                  | (G)<sup>b</sup> (31)     |
|                  | Q(23)                    |
|                  | L(58)                    |
|                  | T(27)                    |
|                  | Y(31)                    |
|                  | V(23)                    |
|                  | M(15)                    |
|                  | K(23)                    |

<sup>a</sup> Residues occurring in more than 15% of 26 Group A peptides from Table III were included in this analysis. The residues encoded by vector sequences were not included in this compilation.

<sup>b</sup> (G) encoded by vector sequences.

**TABLE V**

Catalytic properties of peptides with MMP-14 and MMP-11

| Peptide Number | Sequence | MMP-14 | MMP-11 | MMP-11-A235P | MMP-11-E216A |
|----------------|----------|--------|--------|--------------|--------------|
| MA13           | GGPGG    | 16,000 | <20    | <20          | <20          |
| MA15           | GGKAN    | <200   | 710    | 2,900        | <20          |
| MA18           | GQYAE    | <200   | 2,030  | 20,800       | <20          |
| MA16           | GQKPPQ   | 6,100  | <20    | <20          | <20          |
| MA17           | GQTYDA   | <200   | <20    | <20          | <20          |
| MA20           | GGFLAL   | 7,400  | <20    | 70           | <20          |
| MA21           | GQAGA    | <200   | 70     | 350          | <20          |

<sup>kr</sup>/<i>K<sub>m</sub></sup> values are provided in <i>µ</i>mol<sup>-1</sup> s<sup>-1</sup>.
substances in that it contained Ala in position 3, Ala in position 1, and I/Y in position 1’ but differed from other MMP substrates in that proline was rarely found in position 3 and Asn was frequently found in position 1. A second class with four members contained the sequence G(G/A/E)↓L/R. Although other MMPs cleave peptides with this sequence, most other MMP substrates with this sequence contain proline in position 3. A comparison of the crystal structure for the MMP-8/PLG complex with the mouse MMP-11 structure showed that a loop that is critical for the formation of a pocket for the proline in the MMP-8/PLG complex has a dramatically altered conformation in mouse MMP-11 and, by inference, human MMP-11. Given the magnitude of this structural alteration, it is not surprising that MMP-11 has a different position 3 site preference than other MMPs.

In vitro reactions with peptides confirmed that peptides from both classes were hydrolyzed by MMP-11 and MMP-11-A235P. These in vitro reactions also showed that the $k_{cat}/K_{m}$ values for MMP-11 cleavage of these substrates was modest relative to the values for other MMPs with peptide substrates. In particular, optimal peptide substrates for MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-14 from combinatorial peptide libraries had $k_{cat}/K_{m}$ values with their respective MMPs of 1.6, 82, 6.9, 120, 49, and 6.9 $\text{mM}^{-1}\text{s}^{-1}$ (19). Hence, the $k_{cat}/K_{m}$ values of 0.7 and 2.0 $\text{mM}^{-1}\text{s}^{-1}$ for the digestion of MA15 and MA18, respectively, by MMP-11 were on the low end of the range for known MMP peptide substrates. The ST3 (MMP-11) $k_{cat}/K_{m}$ values reported previously using dansyl-heptapeptides ranged from 1300 to 16,700 $\text{mM}^{-1}\text{s}^{-1}$ (see Table I in Ref. 21). The range of these values is similar to values reported here for wild type MMP-11 using peptide substrates MA15 and MA18 (710 and 2030 $\text{mM}^{-1}\text{s}^{-1}$) and for the mutant MMP-11-A235P (2900 and 20,800 $\text{mM}^{-1}\text{s}^{-1}$) (Table V). Therefore, although the individual $K_{m}$ values reported for ST3 (MMP-11) for two dansyl-heptapeptides are 10–100-fold lower than those reported here (Table VI), our $k_{cat}$ values are 10–100-fold higher (see Table II in Ref. 21). There are two reasonable explanations for these differences. First, the earlier report used mouse ST3 (MMP-11) instead of the recombinant human MMP-11 and the mutant MMP-11 (A235P) used in these studies. Second, the earlier report used dansylated peptides, whereas this study used unmodified peptides. Thus, the 10–100-fold lower $K_{m}$ values reported using the dansyl-heptapeptides could have resulted from solvation entropy effects caused by the addition of the dansyl group to the peptide substrates (26). Interestingly, although the addition of the dansyl group could have increased the apparent affinity (lower $K_{m}$) because of a $+\Delta S$ from solvation entropic effects, the dansyl group could also have resulted in a lower catalytic activity of the enzyme. The higher turnover number could also be attributed to the longer length of the substrate used in these studies. Clearly, further experiments will be needed to test these hypotheses.

A comparison of human MMP-11 with other MMPs showed that alanine 235 is unusual in that most MMPs contain proline at the corresponding residue. Previous work (12) and results from this study showed that MMP-11-A235P was more active than MMP-11. The recent mouse MMP-11 crystal structure offers an explanation for the effect of this mutation on catalytic activity. In particular, this proline forms a turn below the active site that is likely to interact with the substrate. Hence, it is not surprising that mutations that could disturb this turn, such as alanine, decrease catalytic activity. Interestingly, whereas MMP-11-A235P was catalytically more active than MMP-11, the substrate preference appeared unchanged.

The identification of two classes of MMP-11 substrates suggests that subsite preferences for MMP-11 cleavage are de-
peptide sequences derived from the MMP-11 selected phage were used to search the protein data bases for potential MMP-11 substrates. This analysis revealed that many proteins contained the tetrapeptide sequences AANL, GANL, AELR, or GELR. Some proteins identified in these searches were reported substrates for other MMPs, such as perlecan and cell surface-bound Fas ligand, and proteins related to reported MMP substrates, such as pro-transforming growth factor-β1, several protocadherins, acid-labile subunit of the insulin-like growth factor binding protein complex, integrin αι, integrin α5, integrin αm, and integrin β3 (see Ref. 30 for review of potential MMP substrates). A recent paper (31) has identified a novel intracellular isoform of MMP-11 that is translated in its activated form. These provocative results imply the existence of potential intracellular targets for MMP-11 in addition to those extracellular proteins that are the standard fare of MMPs. Consequently, the most interesting protein identified in the search of the data base was the estrogen receptor α, which contains the sequence 288AANL291 in the hinge region separating the DNA-binding domain and the ligand-binding domain. Although the ligand-binding and the DNA-binding domains of estrogen receptor α and β are highly conserved, the hinge domains are not conserved, and the estrogen receptor β lacks this sequence (32). Preliminary in vitro data indicate that only the estrogen receptor α is cleaved by MMP-11. Although these results are intriguing, the large number of proteins identified by this search suggests that only a small fraction are likely to be physiologically relevant MMP-11 substrates. Identifying these substrates will require additional studies.
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Identification of Peptide Substrates for Human MMP-11 (Stromelysin-3) Using Phage Display
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