Biochemical Characterization and N-terminomics Analysis of Leukolysin, the Membrane-type 6 Matrix Metalloprotease (MMP25)

CHEMOKINE AND VIMENTIN CLEAVAGES ENHANCE CELL MIGRATION AND MACROPHAGE PHAGOCYTIC ACTIVITIES*†²

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Amanda E. Starr†¹, Caroline L. Bellac*‡, Antoine Dufour*‡, Verena Goebeler*‡, and Christopher M. Overall*‡§¶

From the †Centre for Blood Research and Departments of §Biochemistry and Molecular Biology and ¶Oral Biological and Medical Sciences, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Background: Neutrophil-specific membrane-type 6 matrix metalloprotease (MT6-MMP)/leukolysin has seven known substrates.

Results: We identified 72 new MT6-MMP substrates by proteomics and family-wide chemokine screens. Cell membrane-bound vimentin chemoattracts macrophages, whereas MT6-MMP-cleaved vimentin is an “eat-me” signal greatly increasing phagocytosis.

Conclusion: MT6-MMP substrates indicate a role for clearance of apoptotic neutrophils.

Significance: MT6-MMP cleaves many bioactive proteins important in innate immunity.

The neutrophil-specific protease membrane-type 6 matrix metalloproteinase (MT6-MMP)/MMP-25/leukolysin is implicated in multiple sclerosis and cancer yet remains poorly characterized. To characterize the biological roles of MT6-MMP, it is critical to identify its substrates for which only seven are currently known. Here, we biochemically characterized MT6-MMP, profiled its tissue inhibitor of metalloproteinase inhibitory spectrum, performed degradomics analyses, and screened 26 chemokines for cleavage using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. MT6-MMP processes seven each of the CXC and CC chemokine subfamilies. Notably, cleavage of the neutrophil chemoattractant CXCL5 activates the chemokine, thereby increasing its agonist activity, indicating a feed-forward mechanism for neutrophil recruitment. Likewise, cleavage also activated CCL15 and CCL23 to increase monocyte recruitment. Utilizing the proteomics approach proteomic identification of cleavage site specificity (PICS), we identified 286 peptidic cleavage sites spanning from P6 to P6 from which an unusual glutamate preference in P1 was identified. The degradomics screen terminal amine isotopic labeling of substrates (TAILS), which enriches for neo-N-terminal peptides of cleaved substrates, was used to identify 58 new native substrates in fibroblast secretomes after incubation with MT6-MMP. Vimentin, cystatin C, galectin-1, IGFBP-7, and secreted protein, acidic and rich in cysteine (SPARC) were among those substrates we biochemically confirmed. An extracellular “moonlighting” form of vimentin is a chemoattractant for THP-1 cells, but MT6-MMP cleavage abolished monocyte recruitment. Unexpectedly, the MT6-MMP-cleaved vimentin potently stimulated phagocytosis, which was not a property of the full-length protein. Hence, MT6-MMP regulates neutrophil and monocyte chemotaxis and by generating “eat-me” signals upon vimentin cleavage potentially increases phagocytic removal of neutrophils to resolve inflammation.

As a critical cell of the innate immune system, neutrophils store components that are required for transendothelial migration and antibacterial and proinflammatory activities (1). In inflammation, neutrophils release proteases, including neutrophil-specific membrane-type 6 MMP³ (MT6-MMP; MMP25; leukolysin) and MMP8 and antimicrobial peptides, they phagocyte pathogens and release soluble mediators, including proinflammatory cytokines and chemokines, to propagate the inflammatory response (2). Short lived, neutrophils die by apoptosis in an MMP8-regulated process (3). Upon display of “eat-me” proteins on the apoptotic cell membrane, phagocytosis by

³ The abbreviations used are: MMP, matrix metalloproteinase; HFL, human fetal lung fibroblast; IGFBP, insulin-like growth factor-binding protein; iTRAQ, isobaric tags for relative and absolute quantitation; MT6-MMP, membrane-type 6 MMP; PICS, proteomic identification of cleavage site specificity; SPARC, secreted protein, acidic and rich in cysteine; TAILS, terminal amine isotopic labeling of substrates; TIMP, tissue inhibitor of metalloproteinase; sMT6, soluble MT6; OF, FRET-quenched fluorescent; Mca, methylcoumarin; Dpa, 2,4-dinitrophenyl; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; m, murine; h, human; IFG, insulin-like growth factor.

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macrophages is promoted (4, 5) as a key event prior to tissue remodeling and resolution of inflammation. However, the mechanisms that target neutrophils for phagocytic clearance are not well understood nor is it clear how eat-me signals are generated from normal proteins.

Cellular recruitment of neutrophils and monocytes is dependent in part upon specific chemotaxing cytokines, termed chemokines, which are produced and released from resident and recruited cells. Of the two main chemokine subfamilies, CXC chemokines primarily recruit neutrophils, whereas CC chemokines are more important in the recruitment of monocytes. Proteolysis of chemokine termini results in significant functional changes (6) with MMP cleavage (7) having importance in modifying cell recruitment and inflammation in vivo (6).

The neutrophil chemotaxing human CXCL8 and CXCL5 and murine CXCL5/LIX are potently activated by stromal MMPs and neutrophil-specific MMP8 (8–10), whereas human CXCL1, -2, -3, -5, -6, -7, and -8 are inactivated by MMP1, -9, and macrophage-specific MMP12 (8). This leukocyte-MMP-directed regulation of neutrophil and monocyte chemokines led us to address the role of the poorly understood neutrophil-specific cell membrane MT6-MMP in processing chemokines for which just seven substrates, mainly extracellular matrix proteins, have been identified in the past 13 years since cloning (11, 12).

MT6-MMP is membrane-associated through a glycosylphosphatidylinositol anchor and contains a furin cleavage sequence for intracellular activation in the Golgi (11, 12). Enzymatic activity of MT6-MMP is regulated by the abundant serum protein clusterin (13) and by the tissue inhibitors of metalloproteinases (TIMPs) 1, 2, and 3 (14–16); notably the role of TIMP4 is unknown, but it is frequently associated with vascular tissue. MT6-MMP is localized primarily in neutrophil gelatinase granules but is also found in specific granules, secretory vesicles, and lipid rafts on the plasma membrane of resting cells (15, 17). Stimulation of neutrophils by CXCL8 and interferon-γ induces MT6-MMP release, whereas stimulation and induction of apoptosis by phorbol 12-myristate 13-acetate relocates MT6-MMP to the neutrophil surface (15, 17), suggesting that the enzyme functions differently at multiple stages of the inflammatory process. MT6-MMP function is implicated in development and disease by increased expression (18), but its few known substrates are limited to the usual ones tested for MMP activity, type IV collagen, gelatin, fibrinectin, fibrin, α1 proteinase inhibitor, urokinase plasminogen activator receptor, and myelins basic protein (14, 19–21), revealing little to distinguish it from other MMPs in its potential in vivo roles.

Identification of the substrate repertoire of a protease (the substrate degradome (22)) is critical to deciphering the biological role of proteases. We recently developed a proteomics approach termed terminal amine isotopic labeling of substrates (TAILS) to specifically enrich for the new N termini (termed neo-N termini) of cleaved substrates from a protease-treated proteome (23). The use of isobaric mass tags for relative and absolute quantification (iTRAQ) enables highly controlled experiments by multiplex mass spectrometry analyses (24). TAILS has enabled identification of many new substrates for proteases (23–25).

To explore the biological roles of MT6-MMP, we expressed and purified a soluble form of MT6-MMP. First, we evaluated the ability of MT6-MMP to cleave both neutrophil and monocyte chemotaxing in a hypothesis-directed approach. Using the human lung fibroblast secretome as a relevant proteome that might be encountered by migrating neutrophils, we then applied TAILS to identify MT6-MMP substrates in a hypothesis-generating proteomics screen. In total, 72 substrates were identified, and 19 new substrates were biochemically confirmed. The results of this research provide insight into the role of MT6-MMP in the potentiation and resolution of inflammation.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Recombinant human MMP1, -2, -3, -8, -9, -12, -13, and soluble MMP14 and recombinant murine TIMP1, -2, and -4 were expressed and purified from mammalian systems (26). Recombinant MMP7 (United States Biochemical Corp.), human vimentin and galectin (R&D Systems), and DQ gelatin (Molecular Probes) were purchased. Chemokines and the small molecule MMP inhibitor marimastat were chemically synthesized, purified, and validated for activity as described (27, 28). Insulin-like growth factor-binding protein (IGFBP)-7 protein and antibody, and cystatin C were kindly provided by Drs. Kaoru Miyazaki (Yokohama City University, Japan) and Magnus Abrahamson (University of Lund, Lund, Sweden), respectively.

Recombinant Human MT6-MMP Protein Expression and Purification—We expressed and purified different soluble (s) forms of MT6-MMP (Fig. 1A), all with a FLAG tag, that are catalytically active upon purification (sMT6-MMP) and could be activated by 1 mM 4-aminophenylmercuric acetate (sMT6-MMPAP) because the furin site had been replaced or that remain catalytically inactive (sMT6-MMP(E234A)). To do so, a HindIII site was introduced at the 5′-end of full-length MT6-MMP cDNA (kindly provided by Dr D. Pei, Guangzhou Institute of Biomedicine and Health, China) using the forward primer 5′-CCGAAAGCTTATGCGGCTGCGCTCCGG-3′. A FLAG tag, EcoRI site, and stop sequence were exchanged with glycosylphosphatidylinositol anchor region residues starting after Gly514 to create sMT6-MMP using the reverse primer 5′-CGGAATTCTACTTTGATCGTCCCTGTTATGTC- ACCAGAGCTCCGGCGGCGG-3′. After PCR (35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s), the gel-purified product was digested with HindIII and EcoRI, gel-purified and then ligated into pGEM-T.

The furin activation site of sMT6-MMP was mutated at RRRRRRR to GGAGAGACTC (loop-in (loop-in region is indicated in bold) in 18 PCR cycles (95 °C for 30 s, 55 °C for 60 s, and 68 °C for 13.5 min). Separately, a catalytically inactive mutant (Glu234 to Ala) was generated using forward 5′-GGGCTGCTCATGTTTGGCCACGCC-3′ and reverse 3′-CCGACAGGTTGCAAGGCGGCGGCGGGGG-5′ primers, pro-
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ducing sMT6-MMP(E234A). All PCR products were confirmed by DNA sequencing.

After ligation into pGW1GH vector (generously provided by J. M. Clements, British Biotech Pharmaceuticals, Oxford, UK), the plasmids were electroporated into CHO-K1 cells. Clonal selection was with mycophenolic acid in DMEM supplemented with 10% Cosmic calf serum, hypoxanthine-thymidine supplement (Invitrogen), and xanthine. Positive expression in clones was screened for in 24-h conditioned medium by Western blots using the M2 αFLAG primary antibody (Sigma).

We devised a protein purification procedure for MT6-MMP that was similar for each construct. After stably transfected CHO-K1 cells reached 90% confluence in mycophenolic acid-containing medium the cells were washed with PBS three times, and the medium was replaced with CHO serum-free medium. Conditioned medium was then collected every 24 h for 5–10 days, clarified by centrifugation at 1500 x g for 10 min, and filtered using a 0.2-μm filter. A green Sepharose column (Sigma) in water was used as a first purification step; following washing, proteins were eluted with a 0.5–1.5 M NaCl gradient. After dialysis against Tris-buffered saline (pH 7.4), samples were loaded onto an αFLAG-agarose column and eluted with 0.1 mM αFLAG peptide (Sigma). Protein purity was confirmed by silver-stained 15% SDS-PAGE and by Western blotting using both M2 αFLAG and rabbit αMT6-MMP (ab39031, Abcam). Proteins were quantified by Bradford assay, and the activity of both sMT6 and sMT6-MMP was screened for in 24-h conditioned medium by Western blots with 10% Cosmic calf serum, hypoxanthine-thymidine supplement, and the medium was replaced with CHO serum-free medium. CHO-K1 cells reached 90% confluence in mycophenolic acid-nitrophenyl (Dpa) substrate, QF24 (Mca-Pro-Leu-Gly-Leu-quenched fluorescent (QF) methylcoumarin (Mca) and 2,4-di-thiopropionate (Pierce) for 2 h at 2 °C. Biotin-labeled prime-side peptide library (prepared from K562 cell (ATCC CCL-243) lysate by trypsic digestion followed by silver-stained 15% SDS-PAGE. Chemokine cleavage assay products were analyzed by MALDI-TOF mass spectrometry (MS) on a Voyager-DE STR (Applied Biosystems) using sinapinic acid matrix (33) and confirmed by TOF mass spectrometry (MS) on a Voyager-DE STR (Applied Biosystems). Wiff files were searched with both Mascot and X! Tandem, the union of which was then used for sequence specificity.

Kinetic Evaluation of Fluorescent Substrates—Enzyme kinetics of sMT6-MMP, sMT6-MMPΔF, and sMT6-MMP(E234A) were determined using QF24 and QF35 (Mca-Pro-Leu-Ala-Dpa-Ala-Arg-NH2), and activity was quantified relative to the full-length chemokine. Cystatin C processing was confirmed by silver-stained 15% SDS-PAGE. Chemokine cleavage was defined to be positive when the mass spectrometry spectra showed a cleavage product with >20% ion intensity of the full-length chemokine. Cystatin C processing was confirmed by silver-stained 15% Tris-Tricine SDS-PAGE. Vimentin, IGFBP-7, galectin-1, and SPARC cleavages were confirmed by silver-stained 15 or 7.5% Tris-glycine SDS-PAGE. Gelatin and casein processing were evaluated by zymography of gels polymerized with 0.2 mg/ml proteins. MMP processing of 25 μg/ml DQ gelatin was evaluated by an increase in fluorescence at an excitation/emission of 495/515 nm on a Polarstar Optima 96-well fluorometer. Data were imported, and K_i values were calculated in Prism (GraphPad).

Cleavage Assays—In vitro native substrate cleavage assays performed at enzyme to substrate ratios of 1:10 (w/w) of chemokines, vimentin, IGFBP-7, cystatin C, galectin-1, and secreted protein, acidic and rich in cysteine (SPARC) by sMT6-MMPΔF were performed in a 10-μl reaction containing 50 mM HEPES, 200 mM NaCl, 5 mM CaCl_2, pH 7.4 for 16 h at 37 °C. Chemokine cleavage assay products were analyzed by MALDI-TOF mass spectrometry (MS) on a Voyager-DE STR (Applied Biosystems) using sinapinic acid matrix (33) and confirmed by silver-stained 15% Tris-Tricine SDS-PAGE. Chemokine cleavage was defined to be positive when the mass spectrometry spectra showed a cleavage product with >20% ion intensity of the full-length chemokine. Cystatin C processing was confirmed by silver-stained 15% Tris-Tricine SDS-PAGE. Vimentin, IGFBP-7, galectin-1, and SPARC cleavages were confirmed by silver-stained 15 or 7.5% Tris-glycine SDS-PAGE. Gelatin and casein processing were evaluated by zymography of gels polymerized with 0.2 mg/ml proteins. MMP processing of 25 μg/ml DQ gelatin was evaluated by an increase in fluorescence at an excitation/emission of 495/515 nm on a Polarstar Optima 96-well fluorometer.

HFL-1 Secretome Preparation—Human fetal lung fibroblast-1 (HFL-1) cells (obtained from Dr. C Roberts, University of British Columbia, Vancouver, Canada) were grown to 90% confluence in DMEM containing 10% fetal calf serum. Cells were washed with PBS three times and then with serum-free medium two times before adding fresh serum-free DMEM. After 16 h, conditioned medium was clarified by centrifugation at 1,500 x g for 10 min and filtered with a 0.2-μm filter. Conditioned medium was concentrated and buffer-exchanged to 50 mM HEPES by ultracentrifugation using 3-kDa cutoff membranes (Amicon). The protein concentration was measured by Bradford assay (Bio-Rad).

TAILS of MT6-MMP—For proteomics screening to discover MT6-MMP native substrates, we used TAILS (23). Active sMT6-MMPΔF or sMT6-MMP(E234A) as control was added to concentrated, serum-free HFL-1 secretome at a 1:250 (w/w) ratio in 50 mM HEPES, 150 mM NaCl, 5 mM CaCl_2 in a 541-μl
volume and incubated for 16 h at 37 °C. To enrich for full-length protein N-terminal and neo-N-terminal peptides, the resulting cleavage products were subjected to TAILS as a two-plex iTRAQ experiment (23, 24). Briefly, guanidine hydrochloride and HEPES were added to the HFL-1 cleavage assay products to a final concentration of 2.5 mM and 250 mM, respectively, to denature proteins. Cysteines were reduced with 1 mM tris(2-carboxyethyl)phosphine at 65 °C and alkylated using 5 mM iodoacetamide. Whole protein iTRAQ labeling from the sMT6-MMPΔF or sMT6-MMP(E234A) digested proteomes was achieved in 50% DMSO with iTRAQ labels 114 and 115, respectively. After 30 min at 25 °C, the reaction was quenched with 100 mM ammonium bicarbonate. Labeled samples were combined and precipitated with 9 volumes of acetone/methanol (8:1, v/v) at −20 °C. The protein precipitate was pelleted by centrifugation at 2,500 × g for 30 min at 4 °C, washed with methanol, and resuspended in 50 mM HEPES for digestion with TrypsinGold (Promega) at a 1:100 enzyme/protein ratio for 16 h with a dendritic polyglycerol aldehyde polymer (Flintbox); incubated under acidic and reductive conditions at 37 °C for 16 h at 37 °C. Complete digestion was confirmed by silver-stained 15% SDS-PAGE. To enrich for N termini, peptides were incubated under acidic and reductive conditions at 37 °C for 16 h with a dendritic polyglycerol aldehyde polymer (Flintbox); internal and C-terminal peptides react to the aldehyde-derivated polymer. N-terminally blocked peptides (due to iTRAQ labeling or natural acetylation or cyclization) were recovered from the unbound fraction by ultracentrifugation using 10,000-kDa-cutoff membranes (Amicon) as described in full (34).

The enriched N-terminome samples were fractionated by strong cation exchange-HPLC using a polysulfoethyl A column (100 × 4.6 mm, 5 μm, 300 Å; PolyLC Inc.) on a 1200 series HPLC (Agilent Technologies). Bound peptides were washed for 15 min in 10 mM potassium phosphate, 25% acetonitrile, pH 2.7. Peptides were eluted over a 22-min gradient to 0.3 M NaCl, then 6 min to 0.4 M NaCl, and 2 min to reach 1 M NaCl. 16 fractions, collected every 1.5 min, were concentrated under vacuum, desalted using C18 OMIX tips, and combined based on HPLC relative peak height. Peptide samples (eight fractions) were analyzed by nanospray LC-MS/MS using a C18 column interfaced with a QStar XL mass spectrometer.

**TAILS Data Analysis**—Acquired MS2 scans were searched by both Mascot (version 2.2.2, Matrix Science) and X! Tandem (July 1, 2007 release) against the human International Protein Index protein database (version 3.69). Search parameters were as follows: semi-Arg-C cleavage specificity with up to two missed cleavages; fixed modifications of cysteine carbamidomethylation and lysine iTRAQ; variable modifications of N-terminal iTRAQ, N-terminal acetylation, and methionine oxidation; peptide tolerance and MS/MS tolerance at 0.4 Da; and scoring scheme of ESL-QUAD-TOF. Search results were statistically modeled using Peptide Prophet and iProphet of the TransProteomic Pipeline (35) (version 4.3, revision 0, Build 200902191420) and Libra for quantification of iTRAQ reporter ion intensities. Each data set included only the peptides with an iProphet probability error rate ≤0.05. Using in-house software, CLIPPER (27), data sets were converted to a common format using ClipperConvert. Mascot and X! Tandem lists were combined for each experiment and analyzed as a single experiment or in tandem by CLIPPER. The data were normalized using a correction factor obtained by analysis of the average log_{2}(ratio) natural N-terminal peptides. Candidate substrates were those having a ratio ≥2 standard deviations from the mean. The highest confidence substrates were proteins identified by the same peptide found in two biological replicate experiments. High confidence substrates were identified by multiple spectra of multiple forms of the peptide in one experiment, whereas candidate substrates requiring biochemical validation were identified by peptides identified by one unambiguous spectrum.

**Transwell Migration Assays**—The chemotactic potential of full-length and MMP-cleaved vimentin and chemokines for THP-1 cells (ATCC) was evaluated by Transwell migration (33). The relative number of cells migrating to the lower chamber was determined by CyQUANT reagent (Invitrogen), and fluorescence was evaluated by excitation/emission at 485/538 nm. The chemotactic index was calculated by the ratio of the relative fluorescence of samples from cells migrating in response to stimuli compared with medium as a control. Experiments were carried out in at least quadruplicate and repeated twice. Statistical significance of cleaved versus full-length vimentin was evaluated by t test.

**Phagocytosis Assay**—Fluoresbrite® microparticles (Polysciences, Inc., Warrington, PA) were incubated with test stimuli in the presence of normal human serum for 30 min at 37 °C and then washed twice with Hanks’ balanced saline solution. THP-1 cells were differentiated with 200 ng/ml phorbol 12-myristate 13-acetate for 24 h. Cells were combined with the coated microparticles for 1 h. Phagocytosis was stopped by the addition of ice-cold Hanks’ balanced saline solution, and the cells were washed free of coated microparticles twice with Hanks’ balanced saline solution, trypsinized, and washed. Phagocytosis of the coated microparticles was analyzed by FACS on a BD FACScanto II with BD FACSDiva software (BD Biosciences). Statistical significance of cleaved versus full-length vimentin was evaluated by t test.

**RESULTS**

**Expression of Recombinant sMT6-MMP, sMT6-MMPΔF, and sMT6-MMP(E234A)**—On SDS-PAGE analysis, the main recombinant sMT6-MMP band electrophoresed at the expected position with some minor bands resulting from autoactivation (Fig. 1B) that, as expected, were absent in the preparation of the inactive sMT6-MMP(E234A). The amino acid sequence^{108}YALSG at the start of the 57-kDa (+DTT) sMT6-MMP(E234A) (Fig. 1B) is the correct start of the catalytic domain, indicating that furin cleavage and removal of the prodomain occurs normally. The lower band copurifying with sMT6-MMP(E234A) in equimolar concentrations was identified by Western blot to be TIMP2 (Fig. 1B), consistent with previous studies that identified TIMP2 complexed with mature MT6-MMP (16).

We also generated sMT6-MMPΔF that has the furin activation site deleted to have a form that can be activated by 1 mM 4-aminophenylmercuric acetate and for increased stability during purification and storage. sMT6-MMPΔF migrated as an ~57-kDa doublet (−DTT) likely representing glycosylation variants (36) and at ~37 and 30 kDa when reduced (Fig. 1B). In the absence of the furin cleavage site, the prodomain was still
removed intracellularly at 100 °C (Fig. 1A). Under reducing conditions, the bands at ~30 kDa have the N-terminal sequence LVSPR352, representing a cleavage within the conditions, the bands at 354. Thus, the disulfide-bonded (Cys317-Cys508) hemopexin domain of sMT6-MMP was made to exchange the catalytic Glu residue to Ala, resulting in the catalytically inactive sMT6-MMP(E234A) protein. Another mutation to sMT6-MMP was made to remove the furin cleavage site (sMT6-MMPΔF). Alternatively, a further mutation to sMT6-MMP was made to exchange the catalytic Glu residue to Ala, resulting in the catalytically inactive sMT6-MMP(E234A) protein. 

### Proteomic Identification of Cleavage Site Specificity

The low activity of MT6-MMP against the conventional substrates commonly used to profile MMPs indicated that these were neither optimal nor natural substrates. Therefore, using a K562 cell proteome tryptic library, we used PICS, which enables the simultaneous detection of prime- and nonprime-side cleavage residues by mass spectrometry (29, 30). In total, 286 cleavage sites were identified from P6 to P6′ that showed a strong preference for Leu at P1′ and Val at P2′ and preferences for Pro/Val at P3 and Ala/Val at P1 (Fig. 2E). After adjusting for the natural amino acid abundance, the IceLogo of the PICS data confirmed this and also revealed preferences for Asn in P1 and P2 (Fig. 2F). Notably, MT6-
MMP has a preference for glutamate at P1, an unusual preference for MMPs. Thus, the reduced activity of MT6-MMP for standard MMP QF substrates is consistent with the different preferences observed by PICS analysis, specifically the preference for Gly at P1 by several MMPs that is not preferred for MT6-MMP but is present in QF24.
**TIMP Inhibition**—English et al. (14) found TIMP2 and TIMP3 to be much stronger inhibitors than TIMP1 of an MT6-MMP form consisting of the catalytic domain alone. However, other groups observed similar inhibition by TIMP1 and TIMP2 of both the catalytic domain and sMT6-MMP forms (13, 16). We found that TIMP1 and the previously untested inhibitor TIMP4 were more potent inhibitors of sMT6-MMP than TIMP2, and all were stronger than the small molecule inhibitor marimastat (Fig. 2G).

**Chemokine Processing by MT6-MMP**—To evaluate the role of the neutrophil-specific MT6-MMP in modulating inflammatory cell recruitment, we evaluated its cleavage specificity for 26 chemokines. Substrate selectivity was observed in that cleavage sites were identified in seven of each of the subfamilies.

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### FIGURE 3. MT6-MMP selectively processes both CXC and CC chemokines.

Human or mouse chemokines (1 μg) were incubated at 37 °C for 16 h with recombinant sMT6-MMPΔF in a 10-μl reaction at an enzyme to substrate ratio of 1:10 (w/w). Cleavage assay products of CXCL (A) or CCL (B) chemokines were visualized on silver-stained 15% Tris-Tricine gels. Cleavages of the chemokines listed were not detectable by MALDI-TOF following incubation at a 1:20 molar ratio with sMT6-MMPΔF at 37 °C for 16 h (n > 2). C, cleavage products were assigned by MALDI-TOF mass spectrometry by comparison of measured with predicted mass to charge ratios (m/z) with +1 charge ionization ([M + H]+). Cleavage sites are indicated by an arrow in the corresponding sequence.

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### TABLE 1. MT6-MMP Cleaves CXC and CC Chemokines

| Chemokine   | Predicted m/z [M+H]+ | Measured m/z [M+H]+ | Sequence                  |
|-------------|----------------------|----------------------|---------------------------|
| CXCL2 (1-73)| 7983                 | 7893                 | APLA TELRCQC              |
| CXCL2 (5-73)| 7541                 | 7523                 |                           |
| CXCL5 (1-78)| 8407                 | 8398                 | AGPAAV LRELRCVCL         |
| CXCL5 (8-78)| 7869                 | 7865                 |                           |
| mCXCL5 (1-92)| 9853                | 9843                 |                           |
| mCXCL5 (5-92)| 9511                | 9501                 |                           |
| mCXCL5 (10-92)| 9056                | 9050                 |                           |
| CXCL6 (1-77)| 8317                 | 8310                 | GUVSVA[V]LTRCTCLVLNRVPKTI[G]LQVFP  |
| CXCL6 (29-77)| 5309                | 5301                 |                           |
| CXCL9 (1-103)| 11721               | 11728                | TPVRKGRGC[...][LL]VLKVRKSRQQSK[...][LL]|
| CXCL9 (9-90)| 10136               | 10140                |                           |
| CXCL12 (1-67)| 7835                | 7832                 | KPV[...][L]VSYRCP[...][L]|
| CXCL12 (5-67)| 7424                | 7421                 |                           |
| CXCL12 (1-72)| 8526                | 8522                 | KPV[...][L]VSYRCP[...][L]|
| CXCL12 (5-72)| 8116                | 8114                 |                           |
| CCL2 (1-76)| 8685                 | 8667                 | QPD[...][N]APVTCC[...][N]|
| CCL2 (5-76)| 8275                 | 8257                 |                           |
| CCL4 (1-69)| 7819                 | 7822                 | APMGSD PPTACC            |
| CCL4 (7-69)| 7260                 | 7261                 |                           |
| CCL7 (1-76)| 8957                 | 8956                 | PVRG[...][L]NSTTCC[...][L]|
| CCL7 (5-76)| 8576                 | 8556                 |                           |
| CCL13 (1-75)| 8851                | 8853                 | QPD[...][N]LPVTCC[...][N]|
| CCL13 (1-72)| 8239                | 8240                 | QNYMK[...][L]HGRK[...][L]AHT  |
| CCL13 (5-72)| 7845                | 7844                 |                           |
| CCL15 (1-92)| 10157               | 10154                | QFINDAETELM[...][L]P[...][L]|
| CCL15 (25-92)| 7447                | 7447                 | SF[...][L]HFAADCC         |
| CCL15 (27-92)| 7212                | 7194                 |                           |
| CCL16 (1-97)| 11202               | 11186                | QPKV[...][L]PEWNP[...][L]|
| CCL16 (5-97)| 10750               | 10741                |                           |
| CCL23 (1-99)| 11367               | 11367                | RVTK[...][L]DAEF[...][L]P[...][L]|
| CCL23 (21-98)| 10191               | 10187                | VLLDR FHTSADCC         |
| CCL23 (26-99)| 8452                | 8449                 |                           |
| CCL23 (26-99)| 8452                | 8449                 |                           |
of CXC and CC chemokines (Fig. 3, A and B), whereas 12 chemokines were not processed by MT6-MMP. Cleavage of human but not murine (m) CXCL2 resulted in a product lacking the four N-terminal residues (Fig. 3C) as is common for many MMP-cleaved chemokines (38). Notably the N-terminal sequence of murine CXCL2, AVVASELR, differs significantly from human. Cleavage was also not observed in the related chemokines CXCL1 and CXCL3, which have Ser-Val residues in place of Pro-Leu at P3-P2 of CXCL2, consistent with PICS data (Fig. 2, E and F). Both human and murine CXCL5 were processed by MT6-MMP at the N terminus. Although mCXCL5(10–92) had not been previously identified, the products hCXCL5(8–78) and mCXCL5(5–92) were shown previously to have increased agonist activity (9, 10, 39, 40). Cleavage was also not observed in the related chemokines CXCL1 and CXCL3, which have Ser-Val residues in place of Pro-Leu at P3-P2 of CXCL2, consistent with PICS data (Fig. 2, E and F). Both human and murine CXCL5 were processed by MT6-MMP at the N terminus. Although mCXCL5(10–92) had not been previously identified, the products hCXCL5(8–78) and mCXCL5(5–92) were shown previously to have increased agonist activity (9, 10, 39, 40).

CXCL6 was cleaved to a product that lacks the first 28 residues; cleavage by MMPs beyond the conserved cysteine residues was the only product also found with eight other MMPs (results not shown). The MT6-MMP C-terminal truncation product CXCL9(1–90) was previously observed following MMP7, -9, and -12 activity (10, 41). Processing of CXCL12 by MT6-MMP showed results consistent with those of other MMPs, removing four N-terminal residues to result in a loss of CXCL12 activity on CXCR4, a switch in receptors from CXCR4 to CXCR3, and increased neurotoxicity (42, 43).

Seven CC chemokines were processed by MT6-MMP (Fig. 3B). The removal of four N-terminal residues from CCL2, CCL7, and CCL13 by MT6-MMP is consistent with the previous findings of processing by multiple MMPs; these products are potent receptor antagonists (7, 8, 38). CCL4(1–69) was cleaved to the product CCL4(7–69), which is known to reduce dimerization (44). CCL15 and CCL23 were cleaved at multiple sites by MT6-MMP in their extended N termini, thereby activating these chemokines (45). Truncation of the first four residues of CCL16 has not been reported previously for any MMP.

TAILS Proteomics Analysis to Identify MT6-MMP Cellular Substrates—Secretomes from HFL-1 cells were incubated either with sMT6-MMPΔF (and labeled with iTRAQ 114) or with the catalytically inactive sMT6-MMP(E234A) as control (and labeled with iTRAQ 115) to identify substrates for MT6-MMP. Samples from two biological replicates (Experiments 1 and 2) were processed by TAILS to purify their N-terminomes (Fig. 4A). From TAILS1, 312 and 224 unique peptides at >95% confidence were identified by Mascot and X! Tandem, respectively, after statistical modeling using the TransProteomic Pipeline. The union yielded 407 unique and high confidence N-terminal and neo-N-terminal peptides (Fig. 4B). From TAILS2, Mascot and X! Tandem searches identified 224 and 166 unique peptides with >95% confidence, respectively, for a combined total of 300 unique identifications (Fig. 4B). 89 peptides were common to both experiments (Fig. 4C, supplemental Fig. S1, and supplemental Table I). Separate CLIPPER analysis, which has extremely stringent requirements for the quality of the spectra used to assign peptides, identified an additional 219 and 112 peptides in the TAILS1 and TAILS2 data sets that were not common to TAILS1 and TAILS2 (Fig. 4C and supplemental Tables II and III).

As expected, the abundance ratios of N-terminal semitryptic peptides derived from the natural N terminus of full-length proteins (±N-terminal methionine or ±signal peptide) are unchanged and equally distributed in both the sMT6-MMPΔF- and sMT6-MMP(E234A)-treated secretomes (supplemental Fig. S1). The distribution of unblocked (now labeled by iTRAQ)

FIGURE 4. Peptide and substrate identification by TAILS analysis. A, schematic overview of the method of the TAILS analysis of conditioned medium from cells treated with sMT6-MMPΔF or sMT6-MMP(E234A) as control (and labeled with iTRAQ 114) or with the catalytically inactive sMT6-MMP(E234A) as control (and labeled with iTRAQ 115) to identify substrates for MT6-MMP. Samples from two biological replicates (Experiments 1 and 2) were processed by TAILS to purify their N-terminomes (Fig. 4A). From TAILS1, 312 and 224 unique peptides at >95% confidence were identified by Mascot and X! Tandem, respectively, after statistical modeling using the TransProteomic Pipeline. The union yielded 407 unique and high confidence N-terminal and neo-N-terminal peptides (Fig. 4B). 89 peptides were common to both experiments (Fig. 4C, supplemental Fig. S1, and supplemental Table I). Separate CLIPPER analysis, which has extremely stringent requirements for the quality of the spectra used to assign peptides, identified an additional 219 and 112 peptides in the TAILS1 and TAILS2 data sets that were not common to TAILS1 and TAILS2 (Fig. 4C and supplemental Tables II and III).
and naturally acetylated termini were similar, ~50% (supplemental Fig. S2), as found previously by TAILS of secretomes (23, 24).

MT6-MMP substrate cleavage results in an iTRAQ ratio >1 (114/sMT6-MMP/2F)/115/sMT6-MMP(E234A)), whereas a ratio <1 indicates cleavage within and loss of the N terminus. For increased confidence in the substrates identified, the value of the cutoff ratio for each experiment was set at $\geq 2$ standard deviations from the mean. Ultimately, 171 unique peptides from 58 proteins met all criteria for inclusion. Proteins that were identified in biological replicate experiments were considered to be the highest confidence candidate substrates of MT6-MMP (Table 1), and those identified by $\geq 2$ different spectra were high confidence substrates (Table 2), whereas those identified by one form of a peptide were considered as candidate substrates of MT6-MMP (Table 3) requiring biochemical validation. Notably, where partially overlapping sequences from multiple cleavages are observed, the site of MT6-MMP processing may be obscured by subsequent aminopeptidase activity as is a limitation of proteomics experiments that evaluate a proteome in the native state.

**Validation of TAILS Candidate Substrates**—In total, 58 substrates were identified by TAILS, and we biochemically confirmed five of these, namely cystatin C, IGFBP-7, galectin-1, vimentin, and SPARC. By SDS-PAGE, the shift of cystatin C following MT6-MMP processing is evident and complete (Fig. 5A). From the TAILS data, cystatin C cleavage occurs at the same site, $^{33}$R↓L, for both MMP2, which we showed reduces its cathepsin inhibitory activity (46). MT6-MMP cleavage of IGFBP-7 was also confirmed (Fig. 5B). IGFBP-7 was previously identified as an MMP-2 substrate by iTRAQ degradomics (47) but was not biochemically confirmed. Related to IGFBP-7, IGFBP-1, -2, -3, -4, and -6 are known MMP substrates (24, 46, 48).

Because galectin-1 is known to be a substrate of MMP2 and -14 (46, 48), we considered it likely that it was also a substrate for MT6-MMP even though it was identified by only one high confidence peptide (Table 3). This was the case with SDS-PAGE analysis of incubations of MT6-MMP and galectin-1, confirming cleavage (Fig. 5C). SPARC was identified previously as a substrate of MMP2, -3, -7, -9, and -13 (48), and we now confirm the same for MT6-MMP (Fig. 5D). Unlike the other substrates validated in *vitro*, the one peptide identified for SPARC was only present in the control digest; that is, the experimental iTRAQ ratio decreased, thereby indicating degradation. Indeed, our analyses revealed complete loss of the full-length SPARC band, confirming degradation rather than cleavage to specific fragments. These analyses again confirm that TAILS is highly predictive for substrate identification even from single peptides. Thus, we expect that the majority if not all of the candidates are bona fide substrates.

**Vimentin Functions as a Chemoattractant and upon MMP Cleavage as a Eat-me Signal for Monocytes**—We identified vimentin as one of the highest confidence substrates of MT6-MMP (Table 1) by 10 different peptides shown on the vimentin sequence in Fig. 6A; three were common to both experiments. We characterized this substrate further. By *in vitro* cleavage assay, we confirmed five of these, namely cystatin C, IGFBP-7, galectin-1, and vimentin (Table 1) by 10 different peptides shown on the vimentin sequence in Fig. 6A; three were common to both experiments.

**TABLE 1**

| Protein                        | Ratio 114/115 | Peptide                          |
|-------------------------------|--------------|----------------------------------|
| Actin                         | 0.66         | 5GFAGDDAPR                       |
|                               |              | 2AVYPSIVGR                      |
|                               |              | 11.14 1051TEAPLNPKNR            |
|                               |              | 4.41 2SLSGTTMYTPGQADR           |
|                               |              | 4.53 2LVYDNSGMCKAGFAGDDAPR      |
|                               |              | 26.19 3VYMGMMQKDSYDGEAQSKR      |
| Collagen type I α-1 chain      | 10.29        | 109GLAPPGESGR                   |
|                               |              | 22.27 1004GGPGAGGPGESGR         |
|                               |              | 31.73 1004GGPGAGGPGESGR         |
|                               |              | 32.57 1004GGPGAGGPGESGR         |
|                               |              | 12.27 1004GGPGAGGPGESGR         |
|                               |              | 0.36 1004GGPGAGGPGESGR          |
|                               |              | 6.55 1273LSQEQENIR              |
|                               |              | 23.76 1273LSQEQENIR            |
| Collagen type I α-2 chain      | 1.42         | 1662CAPGILCPGR                  |
|                               |              | 2.29 1235GAGAATGAR             |
|                               |              | 4.65 1418LNQIETTILTEPSGR        |
|                               |              | 7.94 1418LNQIETTILTEPSGR        |
|                               |              | 23.30 4216GGPMDSVLEEGRG        |
|                               |              | 31.22 4216GGPMDSVLEEGRG        |
|                               |              | 7.14 1794G4GPGPEPGQTPGAPAR     |
|                               |              | 14.84 1418LNQIETTILTEPSGR       |
|                               |              | 28.25 1418LNQIETTILTEPSGR       |
|                               |              | 10.94 1418LNQIETTILTEPSGR       |
| Cystatin C                    | 5.54         | 2617LVGGPMDSVLEEGRG            |
|                               |              | 26.71 LVGGPMDSVLEEGRG          |
|                               |              | 26.35 4216GGPMDSVLEEGRG        |
|                               |              | 7.48 4216GGPMDSVLEEGRG         |
|                               |              | 31.22 4216GGPMDSVLEEGRG        |
| IGFBP-5                       | 0.63         | 1062KFLCGVGR                    |
|                               |              | 2.97 1062KFLCGVGR               |
|                               |              | 0.18 1062KFLCGVGR               |
|                               |              | 0.29 1062KFLCGVGR               |
| IGFBP-7                       | 1.46         | 1062KFLCGVGR                    |
|                               |              | 2.42 1062KFLCGVGR               |
|                               |              | 6.72 1062KFLCGVGR               |
|                               |              | 32.03 1062KFLCGVGR             |
| MT6-MMP                       | 8.38         | 3101E1GFNDIAJR                  |
|                               |              | 36.59 3101E1GFNDIAJR            |
|                               |              | 8.67 3101E1GFNDIAJR            |
|                               |              | 4.41 2551E1GFNDIAJR            |
|                               |              | 23.62 2551E1GFNDIAJR           |
|                               |              | 8.73 2551E1GFNDIAJR            |
|                               |              | 17.25 2551E1GFNDIAJR           |
| Transgelin                    | 0.66         | 19.62 115AKHIAEEADR            |
| Tropomyosin α-4 chain          | 3.56         | 23.62 115AKHIAEEADR            |
| Vimentin                      | 0.54         | 19.62 115AKHIAEEADR            |
|                               |              | 23.62 115AKHIAEEADR            |
|                               |              | 1.52 115AKHIAEEADR             |
|                               |              | 0.30 115AKHIAEEADR             |
|                               |              | 0.65 115AKHIAEEADR             |
|                               |              | 35.77 115AKHIAEEADR            |
|                               |              | 0.39 115AKHIAEEADR             |
|                               |              | 8.83 115AKHIAEEADR             |
|                               |              | 2.69 115AKHIAEEADR             |
|                               |              | 4.21 115AKHIAEEADR             |
|                               |              | 0.71 115AKHIAEEADR             |

* Additional peptide identification in tails1.
* Additional peptide identification in tails2.
we confirmed that vimentin is cleaved not only by MT6-MMP (Fig. 6B) but also by seven other MMPs (Fig. 6C). Interestingly, vimentin is displayed on the surface of apoptotic neutrophils (49), and so we reasoned it may act as an eat-me signal (4).

To explore this potential role of vimentin and MT6-MMP in neutrophil function, we showed in Transwell migration assays that full-length vimentin is a chemoattractant for THP-1 monocytic cells, whereas vimentin cleaved by MT6-MMP (Fig. 6D) or MMP12 (not shown) loses chemoattractant activity. The peak chemotactic response was at 450 nM, which is comparable with that of other intraacellular proteins having chemoattractant activity (50). In contrast, phagocytosis by differentiated THP-1 cells of microparticles coated with cleaved vimentin cut by MT6-MMP (Fig. 6E) or MMP12 (not shown) was increased more than 2-fold. Notably, full-length vimentin showed no activity over controls. Hence, macrophage chemoattraction and phagocytosis of apoptotic neutrophils may be important functions of cell surface-displayed and then neutrophil MT6-MMP-cleaved vimentin.

**DISCUSSION**

With just seven substrates reported in the past 13 years since the discovery of MT6-MMP, its function has remained enigmatic. Given the neutrophil-specific expression, we hypothesized that this cell surface MMP would be involved in the proteolytic regulation of specific inflammatory molecules involved in innate immune processes. For the first time, we found that MT6-MMP is inhibited by the predominantly vascularly expressed TIMP4, and by using chemokine family-wide screens and proteomics analyses, we profiled the active site specificity of MT6-MMP. In identifying 286 cleavage sites by PICS, the active site substrate recognition cleft was mapped to extend from P3 to P6. With 72 new substrates for MT6-MMP discovered, including 14 chemokines that recruit neutrophils and monocytes, the substrate degradome of this MMP has been greatly expanded. Notably, the precise cleavage of the CC and CXC chemokines by MT6-MMP leads to their inactivation or activation, consistent with a role in the regulation of innate immune cell responses. Furthermore, several of the 58 new substrates identified by TAILS are known to contribute to inflammation through actions on leukocyte migration. Indeed, with only 20% of the new substrates identified as being extracellular matrix proteins, rather than being primarily an extracellular matrix degrader, MT6-MMP appears to be mainly involved in bioactive molecule cleavage. Notably, vimentin on

**TABLE 2**

High confidence substrates of MT6-MMP

| Protein | Ratio 114/115 | Peptide                        | No. of spectra |
|---------|--------------|--------------------------------|----------------|
| α-Actinin-1 | 24.56        | 13MOPEDWDR crusher             | 2              |
| Collagen type II α-1 chain | 43.76        | 57GPAGPGAPER crusher           | 2              |
| Collagen type IV α-2 chain | 8.59         | 825GATGFPAGAR            | 2              |
| Collagen type VI α-1 chain | 6.84         | 78GLGFPGLQGR crustase         | 2              |
| Dickkopf-related protein 3 | 20.66        | 461FPGPGSPGAR C-Thr             | 1              |
| Extracellular matrix protein 1 | 32.24        | 651PAGPTPGIDCVDYWKR           | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 5.81        | 651PQIDCVDYWKR              | 1              |
| MMP-1 | 1.71         | 443DPEAGPQDGQXR crustase         | 1              |
| MMP-1 | 16.85        | 497GPPDPGLMER             | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 7.81        | 506GDPLMER            | 1              |
| Proteoglycan C-endopeptidase enhancer 1 | 2.96        | 3100LTEIDCPLKDGTSCR          | 1              |
| Protein-lysine 6-oxidase | 33.89        | 113TIVTSGDEEGR              | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 5.36         | 51VEELMTQHKLKR             | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 4.98         | 232ITWEPDGLMR             | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 7.30         | 20ASEGFTATGQRR            | 3              |
| Peptidyl-prolyl cis-trans isomerase A | 7.88         | 25ADANLEAGNVKETR           | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 8.52         | 172PEGLQAEDCSLNGCENGR        | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 37.52        | 245SSAAAGGLTR              | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 6.11         | 328LEAAYEFADRR            | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 4.75         | 364YSSFFGPR             | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 0.39         | d1AVDGEPLGR             | 3              |
| Peptidyl-prolyl cis-trans isomerase A | 8.52         | 8DIAVDGEPLGR             | 3              |
| Peptidyl-prolyl cis-trans isomerase A | 37.52        | 309PSAPDAPTCPKQCR           | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 4.75         | 59LGSQYQQR              | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 0.39         | 57LSLSQYQQR             | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 2.12         | 22APPAQQQPPQR            | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 2.12         | 54LGSQYQQR              | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 2.12         | 135SVSFADDVR             | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 4.01         | 56AMGALELESR            | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 32.69        | 566MGALERSR            | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 12.27        | 84GIDLGGFDEDAERP           | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 7.94         | 88GDFDERP              | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 7.00         | 246VIVDDQDLEGR            | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 7.49         | 26VIDQQDLGR             | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 6.84         | 170LVIENGDLR            | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 38.06        | 171VIEGDLR             | 2              |
| Peptidyl-prolyl cis-trans isomerase A | 41.62        | 21AEQAEKDQKAEDR           | 2              |
| Tryptophan oximase | 17.45        | 24QAEKDQKAEDR           | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 23.20        | 74AEKKATDAEDAVSLNR         | 1              |
| Peptidyl-prolyl cis-trans isomerase A | 13391        | 115AKHIAEDSRR           | 1              |

* Additional peptide identification in TAILS2.
* Additional peptide identification in TAILS1.

Note: With just seven substrates reported in the past 13 years since the discovery of MT6-MMP, its function has remained enigmatic. Given the neutrophil-specific expression, we hypothesized that this cell surface MMP would be involved in the proteolytic regulation of specific inflammatory molecules involved in innate immune processes. For the first time, we found that MT6-MMP is inhibited by the predominantly vascularly expressed TIMP4, and by using chemokine family-wide screens and proteomics analyses, we profiled the active site specificity of MT6-MMP. In identifying 286 cleavage sites by PICS, the active site substrate recognition cleft was mapped to extend from P3 to P6. With 72 new substrates for MT6-MMP discovered, including 14 chemokines that recruit neutrophils and monocytes, the substrate degradome of this MMP has been greatly expanded. Notably, the precise cleavage of the CC and CXC chemokines by MT6-MMP leads to their inactivation or activation, consistent with a role in the regulation of innate immune cell responses. Furthermore, several of the 58 new substrates identified by TAILS are known to contribute to inflammation through actions on leukocyte migration. Indeed, with only 20% of the new substrates identified as being extracellular matrix proteins, rather than being primarily an extracellular matrix degrader, MT6-MMP appears to be mainly involved in bioactive molecule cleavage. Notably, vimentin on
the cell surface is a chemoattractant for monocytes, and we found that MMP-cleaved vimentin potently increases phagocytosis, suggesting that this contributes to marking apoptotic neutrophils for phagocytic clearance by macrophages.

Neutrophils are among the first cells recruited in inflammation. In this early phase of innate immunity, the predominant neutrophil chemoattractant CXCL8 is produced by macrophages, monocytes, and neutrophils and is activated particularly by neutrophil-specific MMP8 in a feed-forward mechanism (9) and also by MMP9, -12, -13, and -14 (8–10, 26) but strangely not by MT6-MMP. Similarly, a murine functional orthologue, mCXCL5/LIX, is activated by MMP8 and to a lesser extent by MMP9 (9, 10), which only poorly compensates for the lack of MMP8 in the Mmp8−/− mouse (9). Following neutrophil activation by CXCL8 and interferon-γ, polymorphonuclear neutrophils express MT6-MMP on the surface and shed the enzyme in an active, soluble form (15, 17) that corresponds to the sMT6-MMP construct. We identified that MT6-MMP processes and activates the neutrophil chemoattractants CXCL2 and human and murine CXCL5 to the products CXCL2(5–73), CXCL5(8–78), and mCXCL5(5–92, 10–92), which we and others previously showed have enhanced agonist and chemotactic potential over their full-length counterparts (9, 10, 39, 40, 51). These results suggest a discrete role for MT6-MMP in promoting a different phase of neutrophil migration. By acting in a positive feedback manner, MT6-MMP activation of human and murine CXCL5 and human CXCL2 has potential to amplify the initial neutrophil recruitment driven by MMP-8 activation of CXCL8. Pleiotropic effects are also likely because the MT6-MMP cleavage of CXCL6, which was also observed for MMP1, -2, -3, -7, -8, -9, -12, -13, and -14 (not shown), is predicted to inactivate the chemokine by destabilization of the protein core as truncation occurs C-terminally to both partners of the disulfide bridges at CXC.

In addition to neutrophil recruitment, MT6-MMP can promote the recruitment of monocytes. Once recruited to tissue, monocyte receptor expression changes from CCR1high (bind-ing CCL15 and CCL23) to CCR2high (CCL2). Both CCL15 and CCL23 are produced by monocytes and macrophages but are relatively weak CCR1 receptor agonists in their full-length forms. Processing of CCL15 and CCL23 by MT6-MMP results in N-terminus-truncated forms that we have recently shown to exhibit increased agonist activity with increased chemotactic potential (45). This suggests a role for neutrophils, through MT6-MMP, in a positive feed-forward mechanism for the progression of the inflammatory response through increased recruitment of monocytes expressing CCR1. In contrast, CXCL12 and the chemokines CCL2, CCL7, and CCL13 lose agonist activity or become receptor antagonists following N terminus truncation (7, 8, 38, 42) as observed by MT6-MMP proteolysis. Overall, this differential processing of chemokines by MT6-MMP may then halt recruited cells, signaling that they have reached the target site, or terminate continued recruitment as one aspect in the dampening of the inflammatory response.

Neutrophil apoptosis is a final crucial event in the innate immune response, important for chemotaxis and activation of macrophages (4, 5, 52, 53), during which there is also enhanced cell surface expression of MT6-MMP (15). One of the highest confidence substrates we identified was vimentin, which recent studies have shown has a "bona fide" extracellular role as a "moonlighting" protein (54). Vimentin is actively secreted by
macrophages following TNF-α stimulation (55) and is displayed on the surface of apoptotic neutrophils (49). We found that upon loss of chemoattractant activity MMP-cleaved vimentin potently promotes phagocytosis by differentiated THP-1 cells. Hence, vimentin displayed on the cell surface of apoptotic neutrophils and cleaved by MT6-MMP may function as an eat-me signal for macrophage phagocytosis.

A number of cleaved proteins identified by TAILS are components of cell proliferation and collagen production pathways and so may be involved in the early phase of wound healing. These include procollagen C-proteinase enhancer, peptidyl-prolyl cis-trans isomerase A, and IGFBP-5 and -7, the latter of which was biochemically confirmed as a novel substrate. IGFBPs are critical regulators of IGF, sequestering and inhibiting the activity of ~99% of circulating IGF (56, 57); cleavage and inactivation of IGFBPs promote IGF activity. Unbound IGF binds surface-expressed IGF receptor to promote cell growth (58, 59). Hence, the specific cleavage of IGFBP-5 and -7 shown here for the first time by any MMP suggests a role for MT6-MMP in unmasking IGF that may promote healing.

Finally, a number of extracellular matrix-associated proteins were identified as substrates, including fibrillin-1, extracellular matrix protein 1, syndecan-4, and type I, II, IV, and VI collagens; galectin-1 and SPARC were also biochemically validated as new MT6-MMP substrates. The latter two are matricellular proteins with multifunctional roles, including proinflammatory functions and wound healing (60–62). This highlights the
usefulness of unbiased proteomics screens using TAILS to identify protease substrates from even one prime-side-cleaved peptide.

Thus, through hypothesis-directed and hypothesis-generating approaches, we have discovered a large number of new MT6-MMP substrates that shed light on the in vivo role of this enzyme and neutrophil function. Notably, the two polymorphonuclear neutrophil-specific proteases MMP8 and MT6-MMP act in a temporally distinct manner on discrete neutrophil CXC chemokines. This implicates MT6-MMP in the later recruitment of neutrophils through the activation of CXCL2 and CXCL5 and in the progression of the acute inflammatory response toward monocyte recruitment by activation of CCL15 and CCL23. Interstitial roles for MT6-MMP are also suggested wherein monocyte chemoattractants, including vimentin, are cleaved, halting haptotaxis and then stimulating phagocytosis of apoptotic neutrophils. In view of the biological activities of these new MT6-MMP substrates and cleavage products, MT6-MMP can potentially contribute to the complex regulation needed in inflammation and wound healing that is being explored in ongoing studies.

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