Matrix Metalloproteinase Processing of CXCL11/I-TAC Results in Loss of Chemoattractant Activity and Altered Glycosaminoglycan Binding*

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Jennifer H. Cox†, Richard A. Dean‡, Clive R. Roberts§, and Christopher M. Overall§∥

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

The CXCR3 chemokine receptor regulates the migration of Th1 lymphocytes and responds to three ligands: CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC. We screened for potential regulation of T cell responses by matrix metalloproteinase (MMP) processing of these important chemokines. The most potent of the CXCR3 ligands, CXCL11, was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry as a substrate of the PMN-specific MMP-8, macrophage-specific MMP-12, and the general leukocyte MMP-9. The 73-amino acid residue CXCL11 is processed at both the NH₂-terminal truncation results in loss of antagonist activity and heparin binding. However, upon COOH-terminal truncation to position 58 there is loss of antagonist activity and heparin binding. Together this highlights an unexpected site for receptor interaction and that the carboxyl terminus is critical for glycosaminoglycan binding, an essential function for the formation of chemokine gradients in vivo. Hence, MMP activity might regulate CXCL11 tissue gradients in two ways. First, the potential of CXCL11 (5–73) to compete active CXCL11 from glycosaminoglycans might lead to the formation of an antagonistic haptotactic chemokine gradient. Second, upon further truncation, MMPs disperse the CXCL11 gradients in a novel way by proteolytic loss of a COOH-terminal GAG binding site. Hence, these results reveal potential new roles in down-regulating Th1 lymphocyte chemoattraction through MMP processing of CXCL11.

Chemokines are a superfamily of low molecular weight chemoattractant cytokines that function in directing the migration of leukocytes and other cell types in a multitude of processes including development, lymphocyte homing, inflammation, and wound repair (1). Chemokines form haptotactic gradients in vivo through associations with proteoglycan glycosaminoglycans (2). Upon interaction with 7-transmembrane G protein-coupled receptors, chemokines induce a chemotactic response. The expression and secretion of inducible chemokines is stimulated during infection or injury to promote rapid and efficient inflammatory and immune responses. Conversely, dampening of inflammation, a critical event in allowing tissue repair to continue unimpeded and in preventing excessive tissue damage and autoimmunity, is known to involve coordinated down-regulation of chemokine expression (3), receptor internalization (4), scavenger receptors (5), and proteolytic mechanisms of inactivation and conversion to antagonists (6).

Specific and limited proteolysis, termed processing (7), of chemokines results in altered bioactivity with functional consequences such as increased or decreased receptor binding (8), conversion of an agonist to an antagonist (6, 9), shedding of membrane-anchored chemokines (10, 11), and changing receptor specificity (12). Cleavage of proteoglycan core protein also disrupts chemokine gradients formed by chemokine binding to the glycosaminoglycan side chains (13). A variety of proteases are thought to be involved in chemokine cleavage including CD13, also known as aminopeptidase N (14), CD26, also known as dipeptidylpeptidase IV (15, 16), dipeptidylpeptidase-8 (16), neutrophil elastase (17), cathepsin G (18), cathepsins B and D (19), and the matrix metalloproteases (MMPs) (2) (reviewed in Refs. 20–22). Several chemokines are important physiological substrates of MMPs, a family of proteases thought to be associated with extracellular matrix turnover (23), including monocytic chemoattractant proteins CCL2, -7, -8, and -13 (6, 9), the ELR⁺ chemokines CXCL5/ENA-78, CXCL8/IL-8, and murine LIX/mCXCL5 (24–27), CXCL9 and CXCL10 (28), CXCL12/SDF-1α and -β (8, 29), and CX₃CCL1/Fractalkine (10).

MMPs are zinc-dependent extracellular endopeptidases, with most having a carboxyl-terminal hemopexin domain containing exosites for substrate binding (30). MMPs are up-regulated in many pathologies including rheumatoid arthritis (31),

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Multiple sclerosis (32, 33), and tumorigenesis (7, 34), all of which have a chronic inflammatory component. The expression of numerous MMPs by immune cells is commonly thought to promote cellular migration through extracellular matrix degradation and hence removal of physical barriers (23). However, this concept is outmoded following T lymphocyte migration studies in three-dimensional extracellular matrices in the presence of potent protease inhibitors (35), MMP-8 knock-out mice, where deficiency in this neutrophil collagenase did not reduce PMN cell migration in response to activated chemokines in vitro and in vivo (25), and recognition of a multitude of bioactive substrates of MMPs through genetic (23) and proteomic analyses (10, 36, 37).

Interestingly, MMPs are produced by cells of both the innate and adaptive arms of immunity, suggesting pleiotropic roles coordinating rapid host defense and more specific or specialized acquired immunity. Examples include the neutrophil-specific MMP-8, the macrophage-specific MMP-12 (also known as metalloelastase), and MMP-9 (also known as gelatinase B), which is expressed by a variety of leukocytes. Interstitial collagenase (MMP-1) and MMP-7 (also known as matrilysin), an endothelial and epithelial-derived protease, are also expressed by macrophages (20). CD4+ T helper lymphocytes (Th1) are involved in managing humoral and cell-mediated responses through cytokine release and subsequent activation of effector cells. Notably, the gelatinases MMP-2 (also known as gelatinase A) and MMP-9 are preferentially expressed by the Th1 subset and CD4+ migration is reduced in the presence of MMP inhibitors (38). Furthermore, membrane type-1 MMP (MMP-14) is the major physiological activator of MMP-2 (39) and is expressed by monocytes and dendritic cells (40, 41).

The CXCR3 chemokine receptor is preferentially expressed on Th1 cells and has three known ligands: CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC. These chemokines are induced by the pro-inflammatory cytokine interferon-γ in several cell types including neutrophils, monocytes, macrophages, T cells, astrocytes, fibroblasts, and endothelial cells (1). The CXCR3 receptor and its cognate chemokines have been implicated in inflammatory disorders such as multiple sclerosis, rheumatoid arthritis, and allograft rejection, as they are up-regulated in these pathological states, presumably causing enhanced infiltration of CXCR3-expressing Th1 cells (42–44). As such, precise regulation of the CXCR3 ligands is key in preventing excessive Th1 recruitment and the resulting pathology. Previous reports have shown that CXCL10 is cleaved at the carboxyl terminus by furin in the cellular context (45), and that CXCL9 and CXCL10 can be truncated at the carboxyl terminus by MMP-8 and MMP-9 (28), however, no functional changes were found for any of these cleavages. Like many chemokines, CXCL11 is cleaved at the amino terminus after proline 2 by dipeptidylpeptidase IV (46, 47) and aminopeptidase N (14), resulting in loss of agonism, but the propensity of MMPs in processing this chemokine are unknown. Here, we have biochemically screened all CXCR3 ligands for MMP processing by evaluating CXCL9, CXCL10, and CXCL11 as leukocytic-MMP substrates and report that CXCL11 is processed by several MMPs resulting in altered receptor binding and glycosaminoglycan affinity. In particular, CXCL11 is first converted to a CXCR3 antagonist by NH2-terminal processing at positions 4 to 5, but this is lost upon COOH-terminal truncation revealing an unexpected site for receptor binding. Further, proteolytic loss of a COOH-terminal glycosaminoglycan-binding site is a new mechanism that might result in dispersal of chemotactic gradients in vivo and so contribute to regulation of Th1 cell recruitment and cell accumulation.

EXPERIMENTAL PROCEDURES

Chemokine Cleavage Assays—All chemokines and analogues of MMP-cleaved chemokines were synthesized using t-Boc (tertiary butyloxycarbonyl) solid phase chemistry as described previously (48). Recombinant human MMP-1, -2, -8, -9, -12, and soluble MMP-14 (lacking the transmembrane domain) were expressed and purified using standard techniques (49, 50). Human MMP-7 was purchased from U. S. Biochemical Corporation. Enzyme activity was followed by the quenched fluorescence synthetic peptide cleavage assay with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Ala-Arg-NH2 (51).

Chemokine cleavage assays were performed in 50 mM Tris, 200 mM NaCl, 5 mM CaCl2, pH 7.5, with initial screening at an enzyme to substrate ratio of 1:10 (w/w) for 16 h at 37 °C, then assessed at lower enzyme concentrations to establish kinetic parameters, biological efficacy, and relevance. Unlike the other MMPs assayed, MMP-12 showed significant loss of activity through autodegradation in 16-h incubations, therefore kinetic analysis was performed after 2 h. Inhibition experiments were done in the presence of 10 μM EDTA, 10 μM Batimastat, or 10-fold molar excess MMP-8 hemopexin C domain. Assay results were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix and confirmed by 15% Tris-Tricine SDS-PAGE.

Hemopexin C Domain Expression and Binding—MMP-8 linker with hemopexin C domain and an NH2-terminal His tag were cloned into the pGMYX vector and expressed in Escherichia coli BL21 gold cells. Cell lysates were solubilized and protein purified by Ni2+-chelate chromatography followed by Superdex™ 75 gel filtration chromatography (GE Healthcare) yielding a pure 26-kDa product. This was confirmed to be MMP-8 linker and hemopexin C domain by Western blotting with antibody against the MMP-8 hemopexin C domain. Assay results were synthesized using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix and confirmed by 15% Tris-Tricine SDS-PAGE.

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Transfected and Isolated Cells—Human CXCR3-transfected B300-19 cells (52) were kindly provided by B. Moser (Bern, Switzerland) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μM β-mercaptoethanol, and 1.0 mg/ml G418. Human T cells were isolated from healthy volunteers as approved by the University of British
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Columbia Clinical Ethics Review Board. Peripheral blood was drawn into EDTA-treated vacutainers and layered on Ficoll-Paque Plus (Amersham Biosciences), as per the manufacturer’s protocol. Monocytes were removed by adhesion to flask surfaces following 3 h incubation at 37 °C, 5% CO2. Isolated lymphocytes were cultured in RPMI 1640, 2 mM l-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were activated by the addition of 5 μg/ml phytohemagglutinin-P (Sigma) for 3 days followed by 6 to 12 days in the presence of 100 units/ml interleukin-2 (Peprotech). Media was changed every 3 days. Expression of CXCR3 was confirmed by flow cytometry (Beckman Coulter EPICS XL-MCL) using mouse anti-hCXCR3 (R&D Systems, clone 49801).

Calcium Mobilization—CXCR3-transfected B300-19 cells were resuspended at 1 × 10^7 cells/ml in RPMI 1640 media supplemented with 1% fetal bovine serum and incubated with 2 μM Fluo-4-acetoxymethyl ester (Molecular Probes) for 30 min at 37 °C. Cells were washed to remove unincorporated agent and resuspended at 1 × 10^6 cells/ml in Hanks’ balanced salt solution (without calcium or magnesium, Sigma), 20 mM HEPES, 2.5 mM probenecid (Sigma). 3.5 × 10^5 cells were plated per well in 96-well clear bottom fluorescent plates (Nunc) and centrifuged at 1,000 × g for 5 min without braking. Cells were allowed to equilibrate at 37 °C for 15 min prior to addition of ligand. Calcium concentration was monitored by excitation of 485 nm and emission of 520 nm with a Molecular Devices FlexStation II as described previously (53). Calibration was performed by addition of 5 μM ionomycin (Sigma) followed by 1 mM MnCl2 (Fisher Biotech) to determine Fmax and Fmin, respectively. Absolute Ca2+ concentrations were calculated as $K_d \times \frac{[(F - F_{min})/(F_{max} - F)]}{F_{max} - F}$ (54), where the $K_d$ of Fluo-4 was 345 nM, as reported by Molecular Probes. Antagonist experiments were performed by preincubating cells in the presence of CXCL11 (5–73) or CXCL11 (5–58), synthetic analogues of MMP-processed CXCL11 at positions 4–5 (CXCL11 (5–73)) and 58–59 (CXCL11 (5–58)), for 120 s prior to the addition of 3 nM full-length CXCL11, designated CXCL11 (1–73).

CXCR3 Internalization—Internalization of CXCR3 on CXCR3-transfected B300-19 cells was measured following incubation of 5 × 10^5 cells with 10 and 100 nM full-length or truncated CXCL11 for 30 min at 37 °C. Cells were washed three times with ice-cold PBS containing 0.5% bovine serum albumin and incubated for 45 min at 4 °C with 2 μg/ml mouse anti-CXCR3 (R&D Systems, Clone 49801) in PBS, 0.5% bovine serum albumin. Cells were washed three times, then incubated for 45 min at 4 °C with 1:200 diluted sheep anti-mouse IgG-fluorescein isothiocyanate (Sigma) followed by 3 final washes and resuspension in 0.5 ml of PBS. Staining was analyzed by flow cytometry (Beckman Coulter EPICS XL-MCL) and internalization was calculated from the mean fluorescence intensity values relative to untreated cells.

Chemotaxis—Chemotactic migration of CXCR3 transfectants and activated T lymphocytes was performed in a 48-well Boyden chamber across a 5-μm pore size polycarbonate membrane (Neuroprobe). Cells and chemokine were diluted in RPMI containing 20 mM HEPES and 1% bovine serum albumin. To the upper chamber, 2.5 × 10^5 cells were added. The chamber was incubated for 3 h at 37 °C, 5% CO2, then the upper chamber was aspirated and washed twice with distilled water. Inhibition experiments were performed as above except the lower chamber contained 10 nM CXCL11 (1–73) in combination with up to 10 μM of the truncated CXCL11 forms. Following incubation, the contents of the lower chamber were transferred to a MaxiSorp 96-well plate (Nunc) and frozen at −80 °C for at least 2 h. Cell content was determined by CyQUANT analysis, a fluorescent nucleic acid-based cellular quantitation assay, by comparison with a standard curve, according to the manufacturer’s protocol (Molecular Probes). Chemotactic index was calculated as a ratio of cells migrating in response to chemokine compared with buffer control.

Circular Dichroism—CD spectroscopy measurements were made in the far-ultraviolet range from 190 to 260 nm using a JASCO J-810 spectropolarimeter. Full-length and truncated CXCL11 forms, and full-length CXCL8 as a control, were resuspended at 10 μM in nanopure water and measured in a quartz cuvette of 0.1-cm path length at 25 °C. Data are reported as the average of 4 scans with a 1-nm bandwidth. When possible, secondary structure composition was predicted with the SOMCD algorithm (55).

Glycosaminoglycan Binding—CXCL11 binding to fluorescein-heparin (Molecular Probes) was determined using fluorescence polarization analysis on the Polarstar Optima 96-well fluorimeter (BMG) essentially as described previously for other binding proteins (56). Incubations contained 0.1 μM heparin-fluorescein isothiocyanate and chemokines at concentrations of 0 to 2 μM, performed in 100 mM Tris, 150 mM NaCl, pH 7.4, for 1 h at 37 °C. To further assess the effect of MMP truncations of CXCL11 on heparin binding, 0.5 ml of 4 μM chemokine in 10 mM potassium phosphate, pH 7.5, was loaded onto 1-ml Hitrap™ heparin-Sepharose and cation exchange Sepharose columns (GE Healthcare) as described previously (57). Bound CXCL11 and synthetic analogues of MMP-cleaved CXCL11 were eluted using a linear gradient of 0 to 1.5 M NaCl over 30 min at a flow rate of 1.0 ml/min and monitored by in-line absorbance at 215 nm on an AKTA Purifier (Amersham Biosciences). The effect of soluble glycosaminoglycans on CXCL11 cleavage by MMP-8 was assessed in 16-h reactions at 37 °C containing a chemokine to GAG ratio of 1:5 (w/w) and an enzyme to substrate ratio of 1:10 (w/w). Heparan sulfate and chondroitin sulfate A, B, and C were obtained from Seikagaku. Hyaluronan was “Healon” from GE Healthcare.

RESULTS

Selective Processing of CXCL9 and CXCL10 by MMPs—To screen for potential regulation of all CXCR3 ligands by MMPs, recombinant MMP-1, -2, -7, -8, -9, -12, and -14 were incubated in vitro with CXCL9 and CXCL10 at an enzyme-substrate ratio of 1:10 (w/w). Of these MMPs, MMP-7 and -12 had the broadest activity, either cleaving or degrading both chemokines (Fig. 1, A and B). In the case of CXCL9, MMP-7 proteolysis generated a single product with a $m/z$ of 10136 Da ([M + H]+), determined by MALDI-TOF mass spectrometry. Upon deconvolution, this corresponds to a truncation of CXCL9 at positions 90–91 with the cleavage site $^{88}$VLKVRK$^{93}$ (Fig. 1D). The

3All cleavage sites were uploaded to MEROPS, the Peptidase Database.
same COOH-terminal truncation of CXCL9 was observed with MMP-12 incubation, but this cleavage was not as efficient as with MMP-7, as some CXCL9 remained intact.

CXCL10 was partially processed by MMP-12, resulting in truncated forms of 8150 and 7931 Da ([M + H]⁺), corresponding to CXCL10-(1–73) and CXCL10-(1–71), respectively (Fig. 1B). The cleavage sites in CXCL10 were 66SKEMSK74 and 71EMS KR76. Although MMP-7 did not generate stable cleavage products of CXCL10, reproducibly reduced chemokine band intensity indicates that degradation occurred due to MMP-7 activity. Interestingly, protolysis of CXCL9 and CXCL10 was exclusively in the carboxyl-terminal region, as determined by MALDI-TOF mass spectrometry (Fig. 1D). The cleavage products CXCL9-(1–90), CXCL10-(1–71), and CXCL10-(1–73) have been identified previously with MMP-8 and MMP-9 (28) and the latter was found to retain full activity when compared with full-length CXCL10 (45). Hence, the present results expand the repertoire of MMPs that process these CXCR3 chemokines. However, under our assay conditions, and even with high enzyme-substrate ratios, we did not detect cleavage of CXCL9 and CXCL10 by MMP-8 or MMP-9 as previously reported (28).

Selective Processing of CXCL11 by MMPs—Of the CXCR3-binding chemokines, CXCL11 was the most susceptible to MMP cleavage, being significantly processed by MMP-7, -8, -9, and -12 (Fig. 1C). Although MMP-7 appeared to almost entirely degrade CXCL11, one peptide ion peak at 6451 Da ([M + H]⁺) was detected by MALDI-TOF MS following cleavage at 56P KS KQA61, representing a transient cleavage product, CXCL11-(1–58) (Fig. 1D). MMP-12 also degraded CXCL11, but three transient truncations were found generating peptides with m/z of 7779, 6451, and 5928 Da ([M + H]⁺) corresponding to CXCL11-(5–73), -(1–58), and -(5–58) with the NH₂-terminal cleavage at 2PMF KRG7. The neutrophil-specific protease MMP-8 specifically cleaved CXCL11 generating stable 7779, 6524, and 5928 Da ([M + H]⁺) truncations corresponding to CXCL11-(5–73), -(5–63), and -(5–58), respectively. MMP-9 processed the chemokine, generating two cleavage products of 6451 and 5928 Da ([M + H]⁺), which represent CXCL11-(1–58) and -(5–58). Hence, it is evident that several MMPs cleave CXCL11 at residues 4–5 and 58–59, whereas MMP-8 is unique in its cleavage of the 63–64 bond at 61ARLIK66 (Fig. 3, see also Fig. 8A).

Processing of mCXCL11—Murine CXCL11, which is 68% identical to the human form but lacks a proline at position 2, was also examined as a MMP substrate. MMP-1, -7, -8, and -12 cleaved the chemokine (Fig. 2A). Several products were detected by MALDI-TOF mass spectrometry and upon deconvolution were determined to represent NH₂-terminal cleavage at 1FLMFQK6 and C-terminal cleavages at 65ARLMQ66 and 71KNFL RR75 (Fig. 2B). MMP-8 cleaved mCXCL11 at all three sites, whereas MMP-7 and MMP-1 cleaved exclusively at the NH₂ and COOH terminus, respectively (Fig. 3). Notably, no stable truncation products were detected with MMP-12, as substrate degradation was evident.

Kinetic Analysis of CXCL11 Processing—We first screened for cleavage at high enzyme-substrate ratios. Because cleavage alone does not indicate a biologically relevant substrate (7), we assessed the enzyme kinetics of MMP cleavage of CXCL11 (Fig. 4). Based on densitometry analysis, where kcat/Km = (ln2)/(E(t1/2)), the kcat/Km of CXCL11 processing was calculated to be in the range from 96 to 190 M⁻¹ s⁻¹ for MMP-7, -8, -9, and -12 (Fig. 4E). Notably, MMP-7 partially cleaved CXCL11 at the lowest enzyme concentration tested, 16 nM, which corresponds to an enzyme-substrate ratio of 1:800 (mol/mol) (Fig. 4A). However, degradation is apparent at concentrations of 62.5 nM MMP-7 and greater, as was also observed for MMP-12, at con-
cetrations of 1000 nM (Fig. 4D). In control incubations MMP activity against fluorogenic substrate was maintained at 16 h except for MMP-12, which exhibited loss of activity due to autodegradation. Hence, MMP-12 was analyzed at higher concentrations for only 2 h due to the instability observed in longer incubations. Both MMP-8 and MMP-9, which are expressed and secreted by neutrophils, efficiently and precisely cleaved CXCL11 at enzyme concentrations of 125 nM and greater, resulting in both amino- and carboxyl-terminal truncations (Figs. 4B and C, see also 8A), so indicating the potential for Th1 lymphocyte regulation by neutrophil-derived proteases.

Interaction of the MMP-8 Hemopexin C Domain with CXCL11—It is well established that MMPs contain exosites, which are substrate-binding sites outside of the catalytic domain important in dictating substrate specificity and efficiency in cleavage (30, 58). In the case of MMP-8 processing of CXCL11, the addition of 10-fold molar excess MMP-8 hemopexin C domain inhibited the formation of truncation products indicating that CXCL11 cleavage is hemopexin C domain-dependent (Fig. 5A). As positive controls, the hydroxamate MMP inhibitor Batimastat (BB94) and the general met-
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FIGURE 5. Exosite interactions with CXCL11. A, MMP-8 cleavage of CXCL11 (16 h at 37 °C at 1:10 enzyme to substrate ratio [w/w]) was inhibited in the presence of EDTA, Batimastat (BB94), and 10-fold molar excess of the MMP-8 linker and hemopexin C domain (LCD). B, enzyme-linked immunosorbent assay analysis of MMP-8 hemopexin linker-C (L-C) domain binding to the CXCR3 chemokines, immobilized in 96-well plate format. The order of assay analysis of MMP-8 hemopexin linker-C was CXCL11 > CXCL10 > CXCL9.

FIGURE 6. MMP-mediated cleavages of CXCL11 result in loss of agonism and the generation of antagonist activity in calcium mobilization assays. A, representative trace of calcium flux, shown as a burst in relative fluorescence units (RFU) following addition of 800 nM CXCL11 in CXCR3-transfected B300-19 cells loaded with the Fluo-4 calcium indicator reagent. Arrow indicates time of CXCL11 addition. B, dose response of calcium mobilization in CXCR3-transfected B300-19 cells in response to CXCL11-(1–73), -(5–73), and -(5–58). Calcium concentrations were calculated from relative fluorescence based on calibration with ionomycin and MnCl2. C, antagonism of calcium mobilization in CXCR3-transfected B300-19 cells by CXCL11-(5–73) and -(5–58). Cells were preincubated with antagonists for 2 min prior to the addition 3 nM full-length CXCL11. Activity values are reported as a percentage relative to that of uninhibited control cells. D, CXCR3 internalization of CXCR3-transfected B300-19 cells following 30 min incubation of 10 and 100 nM CXCL11 at 37 °C. Detection of cell surface levels of CXCL12 was performed by flow cytometry with anti-CXCR3 monoclonal antibody and mean fluorescence intensities were compared with untreated controls and reported as percentage of CXCR3 levels.

alloprotease inhibitor EDTA also blocked proteolysis. Interestingly, MMP-7 and -12 can process several chemokines yet MMP-7 does not have a hemopexin C domain and MMP-12 autolytically removes this during synthesis. Notably, the hemopexin C domain of MMP-12 has significant antibacterial activities (59), which also likely contributes to the beneficial activities of MMP-12 in innate immunity.

To investigate the molecular basis for the selective interaction of MMP-8 with CXCL11 compared with CXCL9 and CXCL10, where there was no cleavage, the affinity of the CXCR3 ligands for the MMP-8 hemopexin C domain was assessed. Enzyme-linked immunosorbent assay experiments demonstrated that CXCL11 has a significantly higher affinity for MMP-8 hemopexin C domain compared with CXCL9 and CXCL10, which only bound at or below background controls (Fig. 5B). The CXCL11 MMP-truncated forms CXCL11-(5–73) and CXCL11-(5–58) bound with similar affinity to the MMP-8 hemopexin C domain as full-length CXCL11 (data not shown), suggesting that interaction occurs with the central region of CXCL11, distant to the cleavage sites.

Calcium Mobilization of CXCR3-transfected B300-19 Cells—Synthetic analogues of MMP-truncated CXCL11 were tested for activity with human CXCR3-transfected B300-19 cells. At a concentration of 800 nM chemokine, there was a clear loss of activity in the MMP-truncated forms of CXCL11, where calcium mobilization is measured as a burst in relative fluorescence (Fig. 6A). Full-length CXCL11-(1–73) caused a dose-dependent increase in intracellular calcium mobilization with an EC50 of 1 nM, consistent with previous reports (60) (Fig. 6B). In contrast, the truncated forms of CXCL11 had no detectable agonist activity at any concentration tested. Because MMP-cleaved CCL2, -7, -8, and -13 (9) and CX3CL1 (10) are receptor antagonists, we assessed the CXCL11-cleaved analogues as receptor antagonists by calcium mobilization. A 2-min preincubation, shown previously to be sufficient time for receptor interaction (61, 62), with CXCL11-(5–73) resulted in significant inhibition of full-length CXCL11 with a moderate IC50 of 500 nM (Fig. 6C), as observed previously (61). However, the CXCL11-(5–58) truncation had reduced inhibition of calcium mobilization, suggesting that the COOH terminus is involved in binding the CXCR3 receptor. Neither CXCL11-(5–73) nor CXCL11-(5–58) caused CXCR3 receptor internalization as measured by flow cytometry (Fig. 6D), confirming the loss of agonistic activity, whereas 100 nM CXCL11-(1–73) caused ~60% internalization, consistent with previous reports (63).

Chemotactic Migration of CXCR3-transfected B300-19 Cells and Human T Lymphocytes—To confirm the calcium mobilization results, CXCR3-transfected cells and activated T lymphocytes were evaluated for chemotaxis toward full-length and truncated forms of CXCL11. In both cell types CXCL11-(1–73) promoted dose-dependent chemotactic migration as expected (Fig. 7, A and C). However, both CXCL11-(5–73) and CXCL11-(5–58) had minimal activity, even at concentrations as high as 100 nM. Again, the NH2-terminal-truncated CXCL11-(5–73) had antagonistic activity with IC50 values of 100 and 1000 nM for CXCR3-transfected B300-19 cells and T lymphocytes,
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Structural Analysis of Truncated CXCL11—MMP processing of CXCL11 results in the removal of 4 NH₂-terminal amino acids and either 10 or 15 COOH-terminal amino acids by cleavage either before or within the COOH-terminal α-helix (Fig. 8A). This helix is markedly cationic, supporting a role in GAG interactions, as shown in this structural representation based on the published NMR structure (Protein Data bank ID 1RJT) (64). Notably, none of the MMP truncations affect the cysteine residues involved in disulfide formation, hence tertiary protein structure is not expected to be adversely affected. Circular dichroism comparing full-length and truncated CXCL11 suggests that the structural integrity remains intact (Fig. 8B). Interestingly, the overall CD profile of CXCL11 resembles that of CC chemokines with a high percentage of random structure, as indicated by strong negativity at 200 nm (65). CXCL8 was analyzed for comparison with another CXC chemokine and this spectrum of a highly ordered protein correlates with published data (65). The higher degree of disorder in CXCL11, in comparison with other CXC chemokines, has been reported previously by NMR analysis (64). Here the CD spectra for CXCL11-(1–73) and -(5–73) are superimposable and SOMCD analysis predicts 17.9–19.4% α-helix, 27.3–29.9% β-sheets, 11.8–15.6% turns, and 39–39.3% random structure (55). In contrast, the CXCL11-(5–58) spectrum reveals reduced ellipticity at 220 nm, suggesting a loss in α-helical structure as expected, but otherwise is comparable with the spectrum for full-length CXCL11.

Heparin Binding Affinity of CXCL11—The abundance of positively charged amino acids in the carboxyl-terminal α-helix of CXCL11 (Fig. 8A) prompted the prediction that COOH-terminal truncation would result in reduced glycosaminoglycan binding. In a heparin-binding assay, $K_{D}$ values were measured by fluorescence polarization spectroscopy. CXCL11-(1–73) and CXCL11-(5–73) bound to heparin with similar affinity, with binding constants ranging from 0.4 to 0.6 μM (Fig. 9A). In contrast, CXCL11-(5–58) had weakened heparin binding, and as important, showed significantly reduced levels of bound chemokine upon saturable heparin binding. Together this indicates loss of a COOH-terminal glycosaminoglycan-binding site that reduced the overall affinity and amounts of chemokine binding. Consistent with this, CXCL11-(5–58) was also shown to have impaired binding to chondroitin sulfate A (data not shown).

Heparin-Sepharose chromatography was used to refine the characterization of the heparin binding properties of the cleaved forms of CXCL11 in relation to the full-length chemokine. Here again the COOH-terminal truncation had significantly reduced affinity (Fig. 9B), confirming the assessment by fluorescence polarization. CXCL11-(1–73) eluted at 0.74 M NaCl, whereas CXCL11-(5–58) eluted at 0.52 M NaCl. Surprisingly, the NH₂-terminal truncation caused enhanced affinity as respectively (Fig. 7, B and D). Also, the CXCL11-(5–58)-truncated chemokine was only a very weak antagonist, with an IC₅₀ of greater than 10 μM in both cell types. However, it is important to note that this represents a concentration that is biologically unattainable to achieve antagonism.

Structural Analysis of Truncated CXCL11—CXCL11 co-incubated with 10 nM full-length CXCL11. Percentage of maximum fluorescence polarization. CXCL11-(1–73) eluted at 0.74 M NaCl, whereas CXCL11-(5–58) eluted at 0.52 M NaCl. Surprisingly, the NH₂-terminal truncation gave enhanced affinity as compared with another CXC chemokine and this indicates loss of a COOH-terminal glycosaminoglycan-binding site that reduced the overall affinity and amounts of chemokine binding. Consistent with this, CXCL11-(5–58) was also shown to have impaired binding to chondroitin sulfate A (data not shown).
CXCL11-(5–73) eluted at 0.81 M NaCl, significantly higher than the full-length counterpart.

To assess the effect of specificity of interaction versus charge effects alone on chemokine binding properties, an equivalent experiment was performed with a strong cation exchange column. The elutions of CXCL11-(1–73), -(5–73), and -(5–58) occurred at 0.74, 0.77, and 0.46 M NaCl, respectively. Loss of the cationic COOH-terminal α-helix in CXCL11-(5–58) yielded a similar reduction in binding to both the heparin and strong cation exchange columns, in comparison with CXCL11-(1–73), suggesting that the removal of charge is responsible for decreased glycosaminoglycan binding. However, the NH2-terminal truncation in CXCL11-(5–73) appears to enhance specific heparin interactions because the elution shift relative to full-length CXCL11 is greater on the heparin column compared with the strong cation exchange column.

The glycosaminoglycan interaction with the cationic residues of the COOH-terminal peptide59KQARLIKKVERKNF73, forming an α-helix in the chemokine, might block MMP cleavage. The addition of excess heparan sulfate, chondroitin sulfate, and hyaluronan did not affect MMP-8 processing of CXCL11 at position 58–59 and 63–64, before and within the α-helix, respectively, confirmed by MALDI-TOF MS analysis (Fig. 9C). Hence, these data demonstrate that CXCL11 can be processed by MMPs within an α-helix and in the presence of glycosaminoglycans, and that the resulting cleavages alter binding affinity.

**DISCUSSION**

Presented herein is the first instance of chemokine cleavage directly affecting glycosaminoglycan-binding properties. Previously the murine chemokine mCXCL1/KC was shown to be mobilized by MMP-7 cleavage of the core protein of syndecan-1, leading to altered neutrophil chemotaxis, but the chemokine itself was not directly processed (13). Hence, cleavage of the cationic COOH-terminal peptide of CXCL11 and resultant loss of a glycosaminoglycan-binding site represents a novel mechanism of chemokine regulation. We suggest that the epithelial and endothelial MMPs 7 and 9, neutrophil MMPs 8 and 9, and macrophage MMPs 7, 9, and 12 mobilize CXCL11 from proteoglycans with disruption of the haptotactic chemokine gradient predicted (Fig. 10). This would serve to enhance the direct effects on chemokine activity resulting from proteolysis of CXCL11 by these leukocytic MMPs. Amino-terminal processing at Phe4–Lys5 resulted in loss of agonist activity, generation of a CXCR3 antagonist, and disruption of the chemokine gradient (Fig. 10). The in vivo analysis of these effects are now in progress.

Chemokine activity is modulated at several levels including regulated expression and secretion, proteolysis, and the formation of haptotactic gradients in tissue. High affinity interactions with the glycosaminoglycan side chains of proteoglycans are critical for in vivo activity of many chemokines as an immobilization mechanism for cell-surface retention and possibly also for presentation to circulating leukocytes (2). MMP-mediated proteolysis has been shown previously to indirectly influence chemokine gradients, but by a different mechanism. In the case
of MMP-7, knock-out mice demonstrate altered neutrophil infiltration in lung inflammation where neutrophils remain in the interstitium without advancing to the alveolar space (13). This phenotype was attributed to MMP-7-dependent shedding of the syndecan-1 ectodomain complexed with the murine ELR\(^+/\)H11001 chemokine mCXCL1/KC, a process required to direct neutrophils to the site of injury. However, proteolysis of KC did not occur. This mechanism contrasts the potential mobilization of CXCL11, where we demonstrate direct MMP cleavage of the chemokine and loss of a heparin-binding site that also has the potential to disrupt the chemokine gradient.

The basic residues lysine and arginine direct glycosaminoglycan affinity through electrostatic interactions; in CCL5 a BBXB motif has been identified (66), whereas CXCL8 contains several key residues at the COOH terminus (67). Notably, the heparin-binding cationic cradle is rarely a simple consensus sequence, but rather a more complex combination of remote positively charged residues that are clustered in the folded conformation, as illustrated with fibronectin module III-13 (68). We hypothesized that the glycosaminoglycan-binding site of CXCL11 is located in the COOH terminus due to an abundance of basic amino acids and indeed the heparin binding interaction was strongly reduced in CXCL11-(5–58). However, residual binding suggests additional sites of heparin interaction independent of the COOH terminus. Surprisingly, removal of the NH\(_2\)-terminal tetrapeptide FPMF in CXCL11 increased heparin-Sepharose binding, perhaps due to the removal of steric hindrance providing stronger interactions with key basic residues. The enhanced affinity of CXCL11-(5–73) is not completely charge-dependent as the equivalent comparison on a strong cation exchange column showed only a minimal increase, indicating specific heparin interactions.

Despite the location of a glycosaminoglycan-binding site near the site of MMP cleavage between positions 58 and 59, MMP cleavage of CXCL11 was unaltered when interacting with soluble heparan sulfate, chondroitin sulfate, and hyaluronic acid. Hence, these findings show for the first time for any protease that direct proteolysis of a chemokine can alter its glycosaminoglycan-binding properties. The resulting enhanced and reduced affinities, depending on the cleavage site, reveal biphasic regulation of chemokine binding to glycosaminoglycans and hence potential complex modulation of in vivo localization and chemotactic activity that is currently under investigation.

Unlike many other proteases, the cleavage site specificity of MMPs remains largely unpredictable, likely due to less restricted substrate amino acid binding pockets (between S3–S3\(^+/\)H11032 (74)) and the complex role of exosite binding in substrate recognition (58). Peptide substrate libraries have demonstrated an MMP preference for proline at P3 (69, 74), and notably the majority of chemokines contain a proline at residue 2 in the NH\(_2\) terminus. Therefore this proline appears to dictate MMP proteolysis of many chemokines at the characteristic residues 4–5 in the NH\(_2\) terminus. However, examples at other NH\(_2\)-terminal cleavage sites suggest that specificity is not so simple. For instance, CXCL5, which has a proline at position 3, is cleaved by MMPs at Val7–Leu8 and Arg9–Glu10 (25, 27). Interestingly, CXCL9, CXCL10, and CXCL11 share a proline at position 2 and are ~40% identical (60), yet only CXCL11 is susceptible to NH\(_2\)-terminal processing by MMPs, illustrating that additional elements are important, such as exosite interactions. Indeed, only CXCL11 bound the hemopexin C domain of MMP-8, but neither CXCL9 nor CXCL10 did and these were not cleaved. Furthermore, murine CXCL11, which has high sequence identity to the human homolog, contains a leucine in place of proline at position 2 and is processed efficiently at position Met1–Phe8 rather than Phe3–Lys5 by MMP-7 and -8. Nonetheless, the predominant COOH-terminal cleavage of CXCL11 at residues 58–59 also contains a
proline at P3. Hence, this data suggests that Pro is not essential for CXCL11 processing, but rather that it directs cleavage to positions 4–5 in the NH₂ terminus. Interestingly, all three cleavage sites on CXCL11 lie on the same face of the protein in a line indicating potential processivity of cleavage upon chemokine tethering to the hemopexin C domain.

It is unusual that MMP-8 cleaves at position 63-64 within an α-helix, generally considered a proteolytically resistant secondary structure. Potentially, hemopexin C domain interactions open the α-helix, similar to MMP-8 cleavage of native type I collagen (30, 50). Nonetheless, other collagenolytic MMPs 1, 2, and 14 had no activity on any of the human CXCR3 ligands. This reveals specificity of the main leukocytic MMPs for chemokine cleavage and hence an immune cell-specific feedback mechanism regulating chemokine activity. In contrast to the specific and selective cleavages by MMP-8 and MMP-9, processing or degradation of the three chemokines by MMP-7 and MMP-12 represent broad specificity. Interestingly, MMP-7 and MMP-12 share structural similarities in that MMP-7 does not encode a hemopexin C domain (70) and MMP-12 loses the domain upon activation (71). In the case of the CXCR3 ligands, it is probable that MMP-7 and MMP-12 have weak specificity, but high activity, resulting in substrate degradation in contrast to the precise processing by other MMPs. Therefore, it seems likely that exosite interactions of the hemopexin C domain contribute greatly to selective cleavage of substrates.

Amino-terminal truncation of chemokines has been reported to result in conversion of receptor agonists to antagonists (6, 9). CXCL11 truncations have been previously characterized and CXCL11-(4–73) was a potent receptor antagonist, whereas CXCL11-(5–73) had moderate inhibitory properties (61). In addition, CD26-mediated cleavage of CXCL11 generates CXCL11-(3–73), another receptor antagonist (47). Antagonist studies with CXCL11-(5–73) are consistent with previous findings, where moderate IC₅₀ values of 100–1000 nM were observed. Surprisingly, the MMP-mediated COOH-terminal truncation of CXCL11 at residues 58–59 significantly decreased the inhibitory properties of this molecule with IC₅₀ values greater than 10 μM, concentrations that are unlikely to be reached in vivo. The COOH terminus of chemokines is not traditionally associated with receptor-binding properties. Rather, the N-loop following the first two cysteines is the major receptor-binding site that confers receptor specificity. Electrostatic interactions in the 30s loop, the region connected the first two β-strands, are also thought to contribute to receptor affinity (72). Therefore, we have demonstrated an unexpected role for the COOH terminus of CXCL11 in receptor binding.

Amino-terminal CXCL11 processing has been described previously for CD13 (14) and CD26 (46, 47), however, the physiological relevance of these interactions remains unclear, particularly given the clinical use of CD26 inhibitors for diabetes (73). In contrast, the role of MMPs in chemokine processing in vivo is well established with evidence from genetic mouse models and neo-epitope antibodies detecting the MMP-processed forms in human biological samples (6, 25, 29). The murine homolog of CXCL11 lacks a proline at position 2 and hence MMP processing of mCXCL11 is altered, restricting the use of genetic models to further explore this mechanism in animal models. Interestingly, MMPs are the only enzymes known to cleave the COOH terminus of CXCL11, indicating potential synergism between multiple proteases in the precise regulation of this potent T-cell chemoattractant.

Specific and limited proteolysis is emerging as a widespread mechanism of rapid, efficient, and irreversible modulation of both cytokine and chemokine activity (for review, see Refs. 20 and 21). To date, MMPs regulate chemokine function by activation, inactivation, generation of antagonists, shedding from the cell membrane, and cleavage of binding proteins. Our present data demonstrates a new mechanism whereby direct cleavage of a chemokine leads to loss of proteoglycan binding predicted to result in dispersion of the chemokine gradient. Here we have biochemically characterized MMP processing of the CXCR3 ligands, uncovering cleavages that alter receptor activation and binding as well as glycosaminoglycan interactions. These cleavage events have the potential to modulate Th1 cell migration and hence disorders such as multiple sclerosis and rheumatoid arthritis, which are now under study in our laboratory. Neutrophils are implicated in mediating tissue damage in the context of chronic inflammation. However, the abundance of neutrophil-derived MMP-8 and MMP-9 may enable regulation of the adaptive immune response through the fine-tuning of Th1 chemotraction. Furthermore, macrophage proteases MMP-7 and MMP-12 have the potential to dampen the Th1 response through chemokine degradation. The ensuing challenge is to assess these mechanisms in the context of homeostatic and pathological states to decipher the physiological consequences of altering these chemotactic signaling pathways in T cell chemotaxis and immune regulation.

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