Proinflammatory Proteases Liberate a Discrete High-Affinity Functional FPRL1 (CCR12) Ligand from CCL23


*J Immunol* 2007; 178:7395-7404; doi: 10.4049/jimmunol.178.11.7395

http://www.jimmunol.org/content/178/11/7395

**References**

This article cites 58 articles, 25 of which you can access for free at:

http://www.jimmunol.org/content/178/11/7395.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Proinflammatory Proteases Liberate a Discrete High-Affinity Functional FPRL1 (CCR12) Ligand from CCL23

Zhenhua Miao,3* Brett A. Premack,† Zheng Wei,* Yu Wang,* Craig Gerard,‡ Henry Showell, Maureen Howard,* Thomas J. Schall,* and Robert Berahovich*

Most chemokines have been found to bind to and signal through single or highly related chemokine receptors. However, a single chemokine protein, a processed form of the alternatively spliced CCL23 (CKβ8/MPIF-1) gene product, potently engages both the “classical” chemokine receptor CCR1, as well as FPRL1, a type of pattern recognition receptor on innate immune cells. However, the mechanism by which the alternative form of CCL23 is processed is unknown. In this study, we show that proteases associated with inflammation cleave CCL23 immediately N-terminal to the 18-residue domain encoded by the alternatively spliced nucleotides, resulting in potent CCR1 and FPRL1 activity. The proteases also cleave CCL23 immediately C-terminal to the inserted domain, producing a typical CC chemokine “body” containing even further-increased CCR1 potency and a released ~18-aa peptide with full FPRL1 activity but no activity for CCR1. This peptide, which we term SHAAGtide, is by itself an attractant of monocytes and neutrophils in vitro, recruits leukocytes in vivo, and is 50- to 100-fold more potent than all other natural agents posited to act on FPRL1. The appearance of SHAAGtide appears to be transient, however, as the proinflammatory proteases subsequently cleave within the peptide, abolishing its activity for FPRL1. The sequential activation of a transient FPRL1 ligand and a longer-lived CCR1 ligand within a single chemokine may have important consequences for the development of inflammation or the link between innate and adaptive immunity. The Journal of Immunology, 2007, 178: 7395–7404.

In higher organisms, protection from invading pathogens is handled by two distinct yet closely coordinated defense mechanisms—the innate and the adaptive immune responses. Innate immunity is based on receptors that have been selected through evolution to recognize highly conserved and widely distributed features of common pathogens (1, 2). The receptors that recognize these pathogen-associated molecular features are often termed “pattern recognition receptors” or PRRs2 (3).4 These PRRs tend to be expressed by specialized subsets of motile “frontline” innate immune system cells; pathogen recognition by the PRRs generates intracellular signals leading to host cell activation (3, 4). These signals also play a critical role in configuring the subsequent adaptive immune responses (e.g., Th1- vs Th2-regulated effector mechanisms) (5–7). Certain PRRs lead to Th1 responses against intracellular parasites, viruses and bacteria, while others generate Th2 responses against extracellular parasites and allergens (8–10).

Chemokines function to orchestrate immune responses by bringing immune system cells to sites of Ag exposure and inflammation. Recently, it has been appreciated that chemokines might function to coordinate innate immune responses to effect successful adaptive immunity (11–13), although the actual mechanisms have not been defined. Myeloid cells, which include monocytes, macrophages, and certain dendritic cell subsets, are responsive to a number of chemokines (14–18). Several of these chemokines are absolutely required for myeloid cell homing to appropriate secondary lymphoid organs for Ag presentation to lymphocytes (18, 19).

In addition to chemokine receptors, many myeloid cells also express the unrelated chemotactic peptide receptors, N-formyl peptide receptor (fMLP-R or FPR), and its homologs, the “orphan” receptors FPRL1 and FPRL2 (20–24). These receptors induce chemotaxis but can also activate myeloid cells and thereby stimulate their Ag-presenting properties (24–26). FPRL1 was cloned originally from a human phagocyte cDNA library and was characterized by nucleotide homology to FPR, although FPRL1 interacts very weakly with fMLP, the main ligand for FPR (20, 21). FPRL1 has also been reported to act as a functional lipoxin A4 receptor, (27–30), although there is still some debate over this activity (31, 32). More recently, several groups studying FPRL1 have described a broad spectrum of low-affinity pathogen-related peptide and lipid ligands, as well as several high-affinity, but nonnatural, synthetic peptide ligands (24–28, 33–39). The ability of FPRL1 to interact with this broad spectrum of pathogen-related ligands is unusual among G protein-coupled receptors and suggests that FPRL1 may represent a novel type of PRR with the potential for regulating innate immune responses to a number of viral and bacterial pathogens.

In humans, a single gene, CCL23, can give rise to four distinct protein products due to alternative splicing of the third exon and to

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org

*ChemoCentryx, Mountain View, CA 94043; and †Children’s Hospital, Harvard Medical School, Boston, MA 02115

Received for publication January 12, 2007. Accepted for publication March 16, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health National Institute of Allergy and Infectious Diseases Grant 1 U19 AI056690 (to ChemoCentryx).

2 The term CKβ8 has been used as one of the traditional names for the CCL23 protein, although CKβ-1 indicates a variant containing a replacement of Arg8 with an 18-residue peptide, due to alternative splicing of the CCL23 mRNA. Removal of the inhibitory N-terminal domain from CKβ8 and CKβ-1 results in forms beginning with Arg2 (CKβ8) or with the initial methionine of the inserted peptide (CKβ8-1). CCL23α = full-length CKβ8; CCL23α Δ24 = N-terminally truncated CKβ8; CCL23β = full-length CKβ-1; and CCL23β Δ24 = N-terminally truncated CKβ-1.

3 Address correspondence and reprint requests to Dr. Zhenhua Miao, ChemoCentryx, 850 Maude Avenue, Mountain View, CA 94043. E-mail address: zmiao@chemocentryx.com

4 Abbreviations used in this paper: PRR, pattern recognition receptor; fMLP-R or FPR, N-formyl peptide receptor; SAA, serum amyloid A.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
N-terminal processing. The CCL23 cDNA, encoding a 99-residue protein (herein designated CCL23α, initially designated CKβ8, MPIF-1; see review in Ref. 17), was initially isolated from a library derived from human aortic endothelial cells (40). The CCL23α transcript has been reported to be constitutively expressed in liver, lung, pancreas, and bone marrow (40, 41), and CCL23α protein has been detected in synovial fluid from rheumatoid arthritis patients (42). In vitro, the CCL23α protein has chemotactic activity on monocytes and some dendritic cells via the chemokine receptor CCR1 (40, 41). The alternatively spliced form of CCL23, encoding a 116-residue protein termed CKβ8-1 (herein designated CCL23β), was isolated from the myeloid cell line THP-1 (43). The CCL23β mRNA uses a splice acceptor in exon 3, 51 nt upstream of the one used by the CCL23α mRNA, resulting in the replacement of the CCL23α Arg25 residue with the 18-residue peptide MLWRRKIGPQMTLSHAAG (44). CCL23β mRNA expression has been detected in pancreas and skeletal muscle (43), but the protein has heretofore not been detected in human tissue. CCL23α readily undergoes proteolytic processing by inflammation-associated proteases, which remove the N-terminal domain encoded by exon 2 (42) and thereby increase the protein’s potency for CCR1 (29, 42, 44). Reombinant N-terminally truncated forms of CCL23α and CCL23β have been produced and are herein designated CCL23α Δ24 and CCL23β Δ24, respectively.

We previously characterized a number of cell types in the granulocyte, myeloid, and lymphoid lineages for their ability respond to >100 chemotactic stimuli in the chemokine superfamily (our unpublished data). The resulting cell migration assay profile revealed unusual migration-inducing activities among the four CCL23 proteins. All four proteins used CCR1 on monocytes, but CCL23β Δ24 was also an extremely potent neutrophil chemotactant. CCL23β Δ24 was recently shown to be a functional ligand for both CCR1 and FPRL1 (45), which is consistent with our previous observation because neutrophils express FPRL1. However, because CCL23β protein has not been detected yet in vivo, the identity of the natural processed form is still unknown. Moreover, the process by which the full-length CCL23β chemokine, which itself is inactive on FPRL1, is processed to the FPRL1-activating form is unknown. In this study, we report our investigation into the natural activation of CCL23β, including defining the mechanism of processing and the determinants responsible for using CCR1 and FPRL1. The implications of these findings on inflammation and immunity are discussed.

Materials and Methods

Reagents

All chemokines were obtained from R&D Systems. Recombinant CCL23β was a special order produced according to the published sequence (38). The other three CCL23 variants are R&D Systems regular catalog items (catalog nos. 371-MP, 131-M1, and 508-CK).125I-1-CLL3/MIP-1α and 125I-WKYVMVm were obtained from PerkinElmer. Lipoxin A4 was obtained from Tocris Bioscience. CCL23α (1-42) was obtained from American Peptide. Serum amyloid A (SAA) was obtained from PeproTech. MIP-1α, WKYVMVm, WKYVMVM, SHAAGuide (MLWRRKIGPQMTLSHAAG), scrambled peptide version of SHAAGuide, CCL23β 1-42 peptide, and SHAAGuide mapping variants were synthesized by either Phoenix Pharmaceuticals or SynPep.

Cells

Human monocytes were isolated from buffy coats (Stanford Blood Center) using CD14 microbeads (Miltenyi Biotec) and magnetic positive selection. Human neutrophils were isolated from fresh peripheral blood of healthy individuals by gradient centrifugation on Ficoll-Hypaque using standard protocols. Transfectants of L1.2 cells expressing human FPRL1 or CCR1 were generated by standard techniques. L1.2 transfecteds were maintained in RPMI 1640 medium with 10% FBS with 2 mg/ml G-418 and treated with 8 mM sodium butyrate 16 h before use.

Binding assay

Competition binding studies were conducted using monocytes and L1.2-CCR1 or L1.2-FPRL1 cells. Cells were incubated for 3 h at 4°C with 125I-CCL3/MIP-1α (final concentration, ~0.05 nM) or 125I-WKYVMVm (final concentration, ~0.01 nM) in buffer (25 mM HEPES, 140 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, and 0.2% BSA; adjusted to pH 7.1) in the presence of increasing amounts of unlabeled chemokine. Reactions were aspirated onto polyethyleneimine-treated glass fiber filters using a cell harvester (Packard Instrument). Filters were washed twice (25 mM HEPES, 500 mM NaCl, 1 mM CaCl2, and 5 mM MgCl2; adjusted to pH 7.1). Scintillant (MicroSint-10) was added, and the filters were analyzed in a Packard Topcount scintillation counter. Data were analyzed and plotted using Prism (GraphPad Software).

Receptor signaling assay

For individual measurements, cells were loaded with 2 μM indo-1/AM (Invitrogen Life Technologies) in culture medium for 45 min at room temperature, then washed with HBSS and resuspended at 107 cells/ml in HBSS containing 0.1% BSA. Relative cytosolic calcium levels were determined using a Photon Technology International fluorometer (excitation at 350 nm, ratio of dual emission at 400 and 490 nm). For measuring calcium dose responses, cells were analyzed with a Fluorometric Imaging Plate Reader (FLIPR384) (Molecular Devices). Cells were loaded with 5 μM fluo-4 (Invitrogen Life Technologies) in HBSS with 0.1% BSA for 1 h at 37°C, then washed, transferred to black-well 96-well plates, and subsequently excited at 505 nm with emission recorded at 530 nm. Data were analyzed and plotted in arbitrary units of fluorescence using Prism. To ensure that responses were specific to FPRL1, a small, molecule antagonist of human FPRL1, identified from a high-throughput compound library screen, was used. This molecule is noncytotoxic and does not interact with any chemokine receptors.

Chemotaxis assay

L1.2-CCR1 and L1.2-FPRL1 transfectants, monocytes, and neutrophils were collected by centrifugation and resuspended in HBSS with 0.1% BSA. The assays were performed in 96-well ChemoTx microplates (NeuroProbe). Chemokines were diluted in HBSS with 0.1% BSA and added to the lower wells (final volume 25 μl), then 20 μl of cell suspension (5 × 105 cells/ml for monocytes, 2.5 × 106 cells/ml for neutrophils) were added to the polycarbonate filters (5-μm pore size for monocytes and FPRL1 transfectants; 3-μm pore size for neutrophils). After incubation of the plates in a humidified chamber at 37°C for 90 min, the filters were removed, and the cells that migrated into the lower chamber were quantified using the CyQuant Cell proliferation assay kit (Molecular Probes).

Immunohistochemistry

BALB/c and C57BL/6 mice were injected intradermally with 50 μl of sterile saline with or without 2 μg of synthetic SHAAGuide or a scrambled version of SHAAGuide. Six hours later, mice were euthanized, and the skin surrounding the injection site was excised. After fixation in 10% neutral-buffered formalin and processing and embedding in paraffin wax, 5-μm wide sections were either stained with H&E (Sigma-Aldrich) or stained immunohistochemically for neutrophils as follows. The slides were immersed in Target Retrieval solution (DakoCytomation) for 20 min at 90°C, rinsed with water, and immersed in TBS containing 2% BSA for 20 min. The sections were then exposed to rat anti-mouse neutrophil mAb (Accurate Chemical and Scientific) or rat IgG2a isotype control Ab (BD Pharmingen) for 1 h, rinsed with TBS, and exposed to biotinylated goat anti-rat Ig (DakoCytomation) for 30 min. The sections were rinsed with TBS, stained with the ABC-AP and fuchsin reagents (both from DakoCytomation), rinsed with water, and counterstained with hematoxylin for 2 min. After rinsing with water, slides were mounted with Crystalmount (Biomedia).

CCL23β proteolysis

CCL23β, CCL23β Δ24, or a synthetic peptide corresponding to the N-terminal 42 residues of CCL23β (containing the N-terminal and SHAAGuide domains) was incubated with proteases at 37°C for varying amounts of time. Proteases included human mast cell chymase (a gift from Dr. N. Schechter, University of Pennsylvania, Philadelphia, PA), Asp-N endopeptidase, and chymotrypsin (both from Sigma-Aldrich). In addition, chemokines were incubated with supernatants collected from a 6-h culture of human neutrophils in serum-free RPMI 1640 medium with or without PMA (0.1 μg/ml) and ionomycin (1 μg/ml; both from Sigma-Aldrich).
Control reactions included neutrophil supernatant or protease without chemokine. The reactions were then subjected to SDS-PAGE on a 10–20% gradient tricine acrylamide gel (Invitrogen Life Technologies) and stained with colloidal Coomassie blue (Pierce).

## Results

**Proinflammatory proteases transform CCL23β into a FPRL1 ligand**

Recently, we published a study showing that serine proteases involved in inflammation perform N-terminal truncations of four alternative CCR1 chemokines in vitro, activating their potency for CCR1 (42). These “NC6” chemokines include human CCL15/MIP-1α/leukotactin-1 and CCL23α/CKβ8/MPIF-1 and mouse CCL6/C10 and CCL9/MIP-1γ. Although CCL23β/CKβ8-1 contains an 18-residue insertion (termed herein the “SHAAG domain”) between the N-terminal inhibitory domain and the C-terminal chemokine domain of CCL23α (Fig. 1A), we reasoned that the same proteases might also cleave CCL23β as they cleave CCL23α. CCL23β was treated for various times with a panel of human proteases, including chymase, elastase, cathepsin G, chymotrypsin, and Asp-N. In addition, CCL23β was treated for various times with medium collected from a 6-h culture of freshly prepared neutrophils (“PMN sup”) with or without activation by PMA and ionomycin (“act. PMN sup”). By separating the reaction products via SDS-PAGE, processing of CCL23β into smaller fragments was readily detected (Fig. 1B). Early time points of digestion yielded multiple proteolytic fragments (Fig. 1B, left two panels), with the largest of the fragments exhibiting electrophoretic mobility identical to recombinant CCL23β Δ24.
Because this fragment was the largest fragment, by necessity it must have been produced by one of the initial, if not the initial, cleavage events. N-terminal sequencing of this fragment produced by chymase or the activated neutrophil medium indicated that the latter cleaved CCL23/H9252 after Val 21, whereas chymase cleaved CCL23/H9252 after Leu 23 (Fig. 1C).

Longer time points of digestion yielded primarily two fragments (Fig. 1B, right panel). The larger of the fragments was similar in mobility to recombinant CCL23/H9252 lacking the N-terminal and SHAAG domains, whereas the smaller fragment was similar in mobility to an 18-aa synthetic peptide (termed “SHAAGtide”) equivalent to the SHAAG domain. N-terminal sequencing of the larger fragment produced by the proteases indicated that they cleaved the CCL23/H9252-sized fragment immediately C-terminal to the SHAAG domain, either after Phe43 (chymase, cathepsin G) or Thr46 (elastase or the activated neutrophil medium; Fig. 1C). The CCL23/H9252-sized fragment and several other fragments present in the short digestions were minor species or undetectable in the longer digestions, suggesting that these fragments were proteolytic intermediates. Indeed, the conversion of the CCL23/H9252-sized fragment to the CCL23/H9252-sized fragment indicates that the proteases cleaved CCL23/H9252 sequentially: first, N-terminal to the SHAAG domain and then C-terminal to the SHAAG domain. Digestions of recombinant CCL23/H9252 by the proteases yielded the same fragments (data not shown).

To determine whether the processed forms of CCL23/H9252 were active for FPRL1, we measured calcium mobilization in L1.2 cells stably expressing human FPRL1. Full-length CCL23/H9252 was unable to induce substantial calcium mobilization in the L1.2-FPRL1 transfectant, similar to control digestions containing protease in the absence of CCL23/H9252 (Fig. 1D). However, the CCL23/H9252 digestions induced calcium mobilization (Fig. 1D). Digestions of CCL23/H9252 with the activated neutrophil medium were particularly potent, perhaps due to the multiplicity of proteases released during neutrophil degranulation. Specificity for FPRL1 was demonstrated by the inability of the digestions to signal in THP-1 cells pretreated with the CCR1 antagonist CCX634 (Fig. 1E). The CCR1 activity of the chymase digestions was not unsurprising because the cleavage C-terminal to the SHAAG domain

(CCL23/H9252 lacking the N-terminal domain). Because this fragment was the largest fragment, by necessity it must have been produced by one of the initial, if not the initial, cleavage events. N-terminal sequencing of this fragment produced by chymase or the activated neutrophil medium indicated that the latter cleaved CCL23/H9252 after Val21, whereas chymase cleaved CCL23/H9252 after Leu23 (Fig. 1C).

Longer time points of digestion yielded primarily two fragments (Fig. 1B, right panel). The larger of the fragments was similar in mobility to recombinant CCL23α Δ24 (CCL23/H9252 lacking the N-terminal and SHAAG domains), whereas the smaller fragment was similar in mobility to an 18-aa synthetic peptide (termed “SHAAGtide”) equivalent to the SHAAG domain. N-terminal sequencing of this fragment produced by the proteases indicated that they cleaved the CCL23/H9252 Δ24-sized fragment immediately C-terminal to the SHAAG domain, either after Phe43 (chymase, cathepsin G) or Thr46 (elastase or the activated neutrophil medium; Fig. 1C). The CCL23/H9252 Δ24-sized fragment and several other fragments present in the short digestions were minor species or undetectable in the longer digestions, suggesting that these fragments were proteolytic intermediates. Indeed, the conversion of the CCL23/H9252 Δ24-sized fragment to the CCL23/H9252 Δ24-sized fragment indicates that the proteases cleaved CCL23/H9252 sequentially: first, N-terminal to the SHAAG domain and then C-terminal to the SHAAG domain. Digestions of recombinant CCL23/H9252 by the proteases yielded the same fragments (data not shown).

To determine whether the processed forms of CCL23/H9252 were active for FPRL1, we measured calcium mobilization in L1.2 cells stably expressing human FPRL1. Full-length CCL23/H9252 was unable to induce substantial calcium mobilization in the L1.2-FPRL1 transfectant, similar to control digestions containing protease in the absence of CCL23/H9252 (Fig. 1D). However, the CCL23/H9252 digestions induced calcium mobilization (Fig. 1D). Digestions of CCL23/H9252 with the activated neutrophil medium were particularly potent, perhaps due to the multiplicity of proteases released during neutrophil degranulation. Specificity for FPRL1 was demonstrated by the inability of the digestions to signal in nontransfected L1.2 cells (data not shown).

As expected, the proteolytic fragments produced by cleavage between the SHAAG domain and chemokine body were potent CCR1 ligands (Fig. 1E). Calcium mobilization was detected in THP-1 cells, which express CCR1 but not FPRL1, after exposure to the longer digestions of CCL23/H9252 with chymase. Specificity for CCR1 was demonstrated by the inability of the digestions to signal in THP-1 cells pretreated with the CCR1 antagonist CCX634 (Fig. 1E). The CCR1 activity of the chymase digestions was not unsurprising because the cleavage C-terminal to the SHAAG domain

(CCL23/H9252 lacking the N-terminal domain). Because this fragment was the largest fragment, by necessity it must have been produced by one of the initial, if not the initial, cleavage events. N-terminal sequencing of this fragment produced by chymase or the activated neutrophil medium indicated that the latter cleaved CCL23/H9252 after Val21, whereas chymase cleaved CCL23/H9252 after Leu23 (Fig. 1C).

Longer time points of digestion yielded primarily two fragments (Fig. 1B, right panel). The larger of the fragments was similar in mobility to recombinant CCL23α Δ24 (CCL23/H9252 lacking the N-terminal and SHAAG domains), whereas the smaller fragment was similar in mobility to an 18-aa synthetic peptide (termed “SHAAGtide”) equivalent to the SHAAG domain. N-terminal sequencing of this fragment produced by the proteases indicated that they cleaved the CCL23/H9252 Δ24-sized fragment immediately C-terminal to the SHAAG domain, either after Phe43 (chymase, cathepsin G) or Thr46 (elastase or the activated neutrophil medium; Fig. 1C). The CCL23/H9252 Δ24-sized fragment and several other fragments present in the short digestions were minor species or undetectable in the longer digestions, suggesting that these fragments were proteolytic intermediates. Indeed, the conversion of the CCL23/H9252 Δ24-sized fragment to the CCL23/H9252 Δ24-sized fragment indicates that the proteases cleaved CCL23/H9252 sequentially: first, N-terminal to the SHAAG domain and then C-terminal to the SHAAG domain. Digestions of recombinant CCL23/H9252 by the proteases yielded the same fragments (data not shown).

To determine whether the processed forms of CCL23/H9252 were active for FPRL1, we measured calcium mobilization in L1.2 cells stably expressing human FPRL1. Full-length CCL23/H9252 was unable to induce substantial calcium mobilization in the L1.2-FPRL1 transfectant, similar to control digestions containing protease in the absence of CCL23/H9252 (Fig. 1D). However, the CCL23/H9252 digestions induced calcium mobilization (Fig. 1D). Digestions of CCL23/H9252 with the activated neutrophil medium were particularly potent, perhaps due to the multiplicity of proteases released during neutrophil degranulation. Specificity for FPRL1 was demonstrated by the inability of the digestions to signal in nontransfected L1.2 cells (data not shown).

As expected, the proteolytic fragments produced by cleavage between the SHAAG domain and chemokine body were potent CCR1 ligands (Fig. 1E). Calcium mobilization was detected in THP-1 cells, which express CCR1 but not FPRL1, after exposure to the longer digestions of CCL23/H9252 with chymase. Specificity for CCR1 was demonstrated by the inability of the digestions to signal in THP-1 cells pretreated with the CCR1 antagonist CCX634 (Fig. 1E). The CCR1 activity of the chymase digestions was not unsurprising because the cleavage C-terminal to the SHAAG domain

As expected, the proteolytic fragments produced by cleavage between the SHAAG domain and chemokine body were potent CCR1 ligands (Fig. 1E). Calcium mobilization was detected in THP-1 cells, which express CCR1 but not FPRL1, after exposure to the longer digestions of CCL23/H9252 with chymase. Specificity for CCR1 was demonstrated by the inability of the digestions to signal in THP-1 cells pretreated with the CCR1 antagonist CCX634 (Fig. 1E). The CCR1 activity of the chymase digestions was not unsurprising because the cleavage C-terminal to the SHAAG domain
were active for FPRL1, we performed the calcium mobilization assay in the FPRL1 transfectant. The 1-42 peptide was unable to induce substantial calcium mobilization, similar to the control digestions containing protease or medium in the absence of input peptide (Fig. 2C). However, the 1-42 peptide digestions with the proteases and neutrophil medium induced calcium mobilization (Fig. 2C). The SHAAGtide peptide itself was active as well, suggesting that amino acids N-terminal to Met were not necessary for FPRL1 function. The pancreatic protease chymotrypsin was not able to render the 1-42 peptide FPRL1 active, nor was a combination of chymotrypsin and Asp-N, suggesting that chymotrypsin cleaved inside the active Asp-N product, rendering it inactive (Fig. 2D). Specificity for FPRL1 was demonstrated by the inability of the digestions to signal in nontransfected L1.2 cells (data not shown) and by the inability of SHAAGtide to signal in L1.2-FPRL1 cells desensitized by WKYMVM peptide (Fig. 2E). As a further control, CCL23Δ24 was unable to signal in L1.2-FPRL1 cells pretreated with pertussis toxin (Fig. 2F).

**SHAAGtide FPRL1 activity mapping**

To determine the precise sequence involved in FPRL1 activity, variants of SHAAGtide containing N-terminal or C-terminal truncations were synthesized and tested for potency in receptor signaling in the FPRL1 transfectant (Table I). The data indicate that the N terminus, but not the C terminus, is essential for SHAAGtide activity. After removal of the N-terminal methionine from SHAAGtide, its potency was reduced ~10-fold. Removal of the first two N-terminal amino acids (Met-Leu) totally abolished SHAAGtide’s ability to signal through FPRL1. Conversely, removal of the C-terminal three amino residues (Ala-Ala-Gly) reduced the peptide’s potency only 3-fold. We also tested many other naturally occurring FPRL1 ligands, including SAA, β-amyloid protein 42, lipoxin A4, and the bacterial tripeptide fMLP. However, SHAAGtide was significantly more potent and efficacious in the receptor signaling and chemotaxis assays than these other ligands (Fig. 3). In particular, only SAA and the synthetic, nonnatural peptide WKYMVM exhibited EC_{50}s < 1 μM.

**SHAAGtide is as potent for FPRL1 as is CCL23β Δ24 but is inactive for CCR1**

Because SHAAGtide by itself was functionally active for FPRL1, we compared it to CCL23β Δ24 for potency on L1.2-FPRL1 cells

---

**FIGURE 3.** Comparison of FPRL1 ligands. A panel of FPRL1 ligands was tested in calcium mobilization (A) and chemotaxis (B) assays using L1.2-FPRL1 cells. A, Ligands were used at the indicated final concentrations. B, Ligands were used at four different concentrations.

---

**Table I. SHAAGtide activity mapping**

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>EC_{50} in L1.2-FPRL1 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKYMVM</td>
<td>0.2</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSHAG</td>
<td>3.5</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSH</td>
<td>19</td>
</tr>
<tr>
<td>MLWRRKIGPQMT</td>
<td>18</td>
</tr>
<tr>
<td>MLWRRKIGPQMT</td>
<td>65</td>
</tr>
<tr>
<td>MLWRRKIGPQMT</td>
<td>190</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSHAG</td>
<td>210</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSH</td>
<td>Inactive</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSHAG</td>
<td>Inactive</td>
</tr>
<tr>
<td>MLWRRKIGPQMTLSHAG</td>
<td>Inactive</td>
</tr>
<tr>
<td>CCL23α</td>
<td>Inactive</td>
</tr>
<tr>
<td>CCL23α Δ24</td>
<td>Inactive</td>
</tr>
<tr>
<td>CCL23β</td>
<td>Inactive</td>
</tr>
<tr>
<td>CCL23β Δ24</td>
<td>11</td>
</tr>
</tbody>
</table>

*Concentration inducing 50% response in receptor-signaling assay.*

produced a fragment nearly identical to CCL23α Δ24, itself a potent CCR1 ligand (Fig. 1E).

The chemokine domain is not necessary for processed CCL23β FPRL1 activity

To delineate the cleavage fragment(s) within the digestions that were responsible for FPRL1 activity, similar experiments were performed on a 42-aa peptide containing the N-terminal domain and the SHAAG domain but no sequences from the chemokine body. The 1-42 peptide was digested for 10 or 60 min with the proteases or the neutrophil medium. SDS-PAGE analysis of the digestions revealed that the 1-42 peptide was processed into a form of FPRL1 ligands was tested in calcium mobilization (A) and chemotaxis (B) assays using L1.2-FPRL1 cells. A, Ligands were used at the indicated final concentrations. B, Ligands were used at four different concentrations.
by three methods: receptor signaling, chemotaxis, and receptor binding. Because CCL23/H9252/H900424 is also a ligand for CCR1, we also compared SHAAGtide to CCL23/H9252/H900424 on L1.2 cells stably expressing CCR1. We included CCL23/H9251/H900424 in our analyses because this chemokine is equivalent to CCL23/H9252/H900424 lacking the SHAAG domain. In calcium mobilization assays, L1.2-CCR1 cells responded to CCL23/H9251/H900424 and CCL23/H9252/H900424, as expected; however, SHAAGtide was inactive (Fig. 4A). In the FPRL1 transfectant, SHAAGtide and CCL23/H9252/H900424 induced signaling, whereas CCL23/H9251/H900424 was inactive (Fig. 4B; also see Table I). A control peptide equivalent to SHAAGtide but with the amino acids in a scrambled order was inactive in the assays, indicating that SHAAGtide activity was sequence specific. In chemotaxis assays, L1.2-CCR1 cells migrated to CCL23/H9251/H900424 with an EC50 of 1.3 nM; CCL23/H9252/H900424 was 10-fold less potent and SHAAGtide was inactive (Fig. 4C). In the FPRL1 transfectant, SHAAGtide and CCL23/H9252/H900424 were equipotent, whereas CCL23/H9251/H900424 was inactive (Fig. 4D). In radiolabeled ligand-binding assays, CCL23/H9252/H900424 induced the binding of the CCL3/MIP-1α tracer to the L1.2-CCR1 cells; consistent with the calcium mobilization and chemotaxis assays, SHAAGtide was unable to bind to the L1.2-CCR1 cells (Fig. 4E). To detect FPRL1 binding, competition was performed with radiolabeled WKYMVM, a potent synthetic ligand for FPRL1. SHAAGtide and CCL23/H9252/H900424 were equipotent in their ability to inhibit the binding of the WKYMVM tracer to the FPRL1 transfectant (Fig. 4F). Collectively, these data indicate the FPRL1-using determinant of CCL23/H9252/H900424 is located entirely within the SHAAGtide domain, whereas the CCR1-using determinant lies entirely within the chemokine domain C-terminal to the SHAAGtide domain.

SHAAGtide is a potent functional ligand for human monocytes and neutrophils

Because SHAAGtide was equipotent with CCL23/H9252/H900424 on the FPRL1 transfectant, the two ligands were compared for their abilities to induce calcium mobilization in freshly isolated human monocytes and neutrophils. Both cell types express FPRL1 and CCR1, but the CCR1 on neutrophils is poorly responsive. As seen with the FPRL1 transfectant, both SHAAGtide and CCL23/H9252/H900424 induced robust receptor signaling in monocytes and neutrophils, whereas the scrambled SHAAGtide was inactive (Fig. 5, A and B). The potent CCR1 ligand CCL15/H9252/H900424/leukotactin was unable to desensitize monocytes or neutrophils to SHAAGtide-induced calcium mobilization (data not shown), indicating that SHAAGtide signaling activity was not mediated by CCR1.
In addition to the receptor signaling assays, chemotaxis assays were performed with both cell types. CCL23Δ24 and SHAAGtide exhibited identical potencies, both in monocytes and neutrophils, while the scrambled peptide was ineffective (Fig. 5, C and D). Chemotaxis assays were performed in monocytes and neutrophils, with three concentrations of ligand. E and F, Recruitment of leukocytes by SHAAGtide in vivo. E, Micrographs show H&E staining of the subcutis region of mouse skin 6 h after intradermal injection of 2 μg of SHAAGtide or an equivalent volume of saline. Magnification: ×100. F, Micrographs show immunohistochemical staining (red color) of the SHAAGtide-injected skin with an Ab specific for mouse neutrophils and an isotype control Ab. Nuclei were counterstained blue with hematoxylin. Magnification: ×400.

Both CCL23Δ24 and SHAAGtide were able to induce calcium mobilization and chemotaxis in murine bone marrow neutrophils (data not shown), suggesting that both ligands can function through a mouse receptor counterpart. To determine whether SHAAGtide was functional in vivo, we injected it intradermally into C57BL/6 and BALB/c mice and analyzed the cells recruited to the site of injection 6 h later by histology. In both strains of mice, a 2-μg dose of SHAAGtide resulted in the consistent (six of six mice) recruitment of leukocytes into the subcutis region of the dermis (Fig. 5E). By immunohistochemistry, a large proportion of the recruited cells were identified to be neutrophils (Fig. 5F). Taken together, these data clearly indicate that the unique SHAAG domain of CCL23 acts as a full agonist for monocytes and neutrophils in vitro and in vivo.

**FPRL1 activity is transient**

Interestingly, extended digestions of CCL23 or the 1–42 peptide with the activated neutrophil medium resulted in less calcium mobilization in the FPRL1 transfectant, compared with shorter digestions (Fig. 6, A and B). SDS-PAGE analysis of these long-term

---

**FIGURE 5.** Functional characterization of CCL23Δ24 and SHAAGtide. A and B, Receptor signaling assays were performed in monocytes and neutrophils with 100 nM ligand. C and D, Chemotaxis assays were performed in monocytes and neutrophils, with three concentrations of ligand. E and F, Recruitment of leukocytes by SHAAGtide in vivo. E, Micrographs show H&E staining of the subcutis region of mouse skin 6 h after intradermal injection of 2 μg of SHAAGtide or an equivalent volume of saline. Magnification: ×100. F, Micrographs show immunohistochemical staining (red color) of the SHAAGtide-injected skin with an Ab specific for mouse neutrophils and an isotype control Ab. Nuclei were counterstained blue with hematoxylin. Magnification: ×400.

**FIGURE 6.** Inactivation of FPRL1 activity. A, The L1.2-FPRL1 calcium mobilization assays were performed with CCL23Δ24 digested with activated neutrophil medium for 4 and 18 h. Note the diminution of calcium mobilization in the 18-h time point compared with the 4-h time point. B, The L1.2-FPRL1 calcium mobilization assay was performed with CCL23Δ24 1–42 peptide digested with activated neutrophil medium for 1 and 4 h. Note the diminution of calcium mobilization in the 4-h time point compared with the 1-h time point. C, Sequence of the 1–42 peptide, containing the N-terminal and SHAAG domains of CCL23Δ24, with the latter underlined. Sites of cleavage of the 1–42 peptide by chymase, the neutrophil medium, and tryptase are indicated. D, L1.2-FPRL1 cells were exposed to 100 nM 1–42 peptide after 1-h digestion with chymase, tryptase, or a combination of the two, and calcium mobilization was monitored over time.
digestions no longer contained a SHAAGtide-sized band, presumably due to protease cleavage within the SHAAGtide. Inspection of the SHAAG sequence revealed putative tryptase cleavage sites near the N terminus critical for FPRL1 activity (Fig. 6C and Table I). Addition of recombinant tryptase to the digestion of the 1-42 peptide with chymase resulted in complete abrogation of the FPRL1 activity (Fig. 6D). N-terminal sequencing of the 1-42 peptide after tryptase exposure indicated that the enzyme indeed cleaved at the putative substrate site (Fig. 6C). Although tryptase is a product of mast cells and not neutrophils, the latter might contain proteases (e.g., matrix metalloproteinasises) that cleave inside the SHAAG domain, similar to tryptase.

**Discussion**

In this article, we demonstrate that proteases associated with inflammation cleave CCL23β/CKβ8-1 (43) into potent CCR1 and FPRL1 functional ligands. The FPRL1-using determinant is located solely within the 18-residue “SHAAG” domain encoded by the alternately spliced nucleotides, whereas the CCR1-using determinant is located solely within the C-terminal chemokine domain. This 74-aa domain is characteristic of the other CC chemokines, including CCL3/MIP-1α, in that four cysteines are placed in conserved positions and there is no inhibitory N-terminal domain. The role of the extra two cysteines in the chemokine domain of CCL23β is unknown, but these extra cysteines are found in the other NC6 chemokines (42) and thus may be used to hold the N-terminal inhibitor domain in place until the chemokine is exposed to proteases. In contrast, the FPRL1-using “SHAAG” domain is completely novel within the chemokine family and allows processed CCL23β to use a chemotactrant receptor outside the chemokine receptor family. Due to the inhibitory N-terminal domain of CCL23β, both CCR1 and FPRL1 functionality are blocked until the chemokine is processed by proteases associated with inflammation.

Human mast cell chymase, neutrophil cathepsin G and elastase, lysosomal protease Asp-N, and human neutrophil-conditioned medium were all found to remove the N-terminal domain from CCL23β, activating the chemokine’s FPRL1 activity through exposure of the SHAAG domain. The proteases subsequently cleaved CCL23β between the SHAAG and chemokine domains, activating the chemokine’s CCR1 activity and also releasing a short peptide (“SHAAGtide”) containing the SHAAG domain. Remarkably, SHAAGtide by itself was functional for FPRL1, with potency and efficacy identical to its parent (N-terminally truncated CCL23β) and to recombinant CCL23β Δ24 (CCL23β lacking the entire N-terminal domain). With EC₅₀ of 10–30 nM on human monocytes and neutrophils, SHAAGtide is by far the most potent naturally existing ligand for FPRL1 identified to date. SHAAGtide is also the most efficacious (albeit not potent) naturally occurring chemotactrant of monocytes and neutrophils we have tested in vitro, causing more chemotaxis than, for example, CXCL8/IL-8 and CCL2/MCP-1. SHAAGtide not only has potent signaling and chemotactrant properties in vitro, it is functional in vivo, recruiting leukocytes, including neutrophils, to mouse skin after intradermal injection.

Mapping of SHAAGtide indicated that the N terminus is critical for FPRL1 activity. Short N-terminal extensions are tolerated because naturally processed forms contain extensions of one to three amino acids and are active on FPRL1. Deletion of the N-terminal methionine, however, reduces SHAAGtide activity for FPRL1 at least 10-fold. Another group recently produced a SHAAGtide lacking the N-terminal methionine and observed weak activity for FPRL1 (45). In contrast, the C terminus of SHAAGtide is relatively unimportant for FPRL1 activity, as deletion of the C-terminal 3 aa did not affect the peptide’s potency on FPRL1-bearing cells.

The ability of CCL23β to function through both CCR1 and FPRL1 suggests that this chemokine might play multiple roles in innate and adaptive immunity. CCR1 controls the trafficking of monocytes, dendritic cells, and effector lymphocytes to sites of chronic inflammation, such as seen in rheumatoid arthritis and multiple sclerosis (46–49). In addition, it is notable that some CCR1 ligands are capable of skewing adaptive immune responses to certain diseases or pathogens (11, 12). FPRL1 functions in the innate immune response by enabling monocytes and neutrophils to recognize with low affinity a variety of proinflammatory and pathogen-associated ligands, including HIV coat proteins, bacterial peptides, Helicobacter coat proteins, serum amyloid-related stress proteins, and host- and pathogen-produced eicosanoids (25, 26, 31, 32, 50–58). Our discovery that FPRL1 can also function as a high-affinity chemokine receptor for CCL23β cleavage products suggests that they might function to induce migration of different sets of leukocytes to sites of infection, whereupon the cells would recognize the pathogenic pattern. Depending on the proteases present, CCL23β could recruit monocytes and neutrophils through FPRL1 (i.e., cleavage N-terminal to the SHAAG domain) or monocytes, dendritic cells, and effector lymphocytes through CCR1 (i.e., cleavage C-terminal to the SHAAG domain). As our data suggest, it is entirely possible that stepwise processing occurs, i.e., cleavage first N-terminal and then C-terminal to the SHAAG domain, producing activity first for FPRL1 and then for CCR1 (Fig. 7).

Whatever the case, our results indicate that the FPRL1-using activity might be transient: some time after the N-terminal inhibitor domain was removed from the SHAAG domain, subsequent cleavage events occurred inside SHAAGtide. The liberated fragment became too small to be detected by SDS-PAGE, and the activity on FPRL1 was diminished. This phenomenon occurred when the substrate chemokine was either full-length CCL23β or a
42-residue peptide containing only the N-terminal and SHAAg domains. Perhaps removal of the N-terminal domain reveals a naturally self-limiting regulation of the activation of this important receptor, which we propose be designated CCR12 in all future nomenclature.

In conclusion, we have provided clear data surrounding the liberation of high-affinity chemokine and chemokine-derived peptide ligands for FPRL1 by naturally occurring proteases. These proteases are apt to constitute a naturally self-limiting regulation of the activation of this important receptor, which we propose be designated CCR12 in all future nomenclature.

Disclosures
The authors have no financial conflict of interest.

References


