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# Proteolytic processing and inactivation of CCL2/MCP-1 by meprins

Christian Herzog [a,b], Randy S. Haun [a,c], Sudhir V. Shah [a,b], Gur P. Kaushal [a,b,d,*]

[a] Central Arkansas Veterans Healthcare System, Little Rock, AR, USA
[b] University of Arkansas for Medical Sciences, Department of Internal Medicine, Little Rock, AR, USA
[c] University of Arkansas for Medical Sciences, Department of Pharmaceutical Sciences, Little Rock, AR, USA
[d] University of Arkansas for Medical Sciences, Department of Biochemistry, Little Rock, AR, USA

A B S T R A C T

Monocyte chemotactic protein 1 (CCL2/MCP-1) is a small chemokine involved in the recruitment and trafficking of mononuclear immune cells to inflammation sites. Our studies demonstrate that the metalloendopeptidases meprin A (purified from kidney cortex), recombinant meprin α, and recombinant meprin β can all process CCL2/MCP-1. The cleavage sites were determined by amino acid sequencing and mass spectrometry analysis of the generated products, and the biological activity of the products was evaluated by chemotactic migration assay using THP-1 cells. The cleavage sites generated by the meprin isoforms revealed that meprin A and meprin α cleaved the N-terminal domain of mouse CCL2/MCP-1 at the $Asn^6$ and $Ala^7$ bond, resulting in significant reduction in the chemotactic activity of the cleaved CCL2/MCP-1. Meprin β was unable to cleave the N-terminus of mouse CCL2/MCP-1 but cleaved the C-terminal region between $Ser^{74}$ and $Glu^{75}$. Human CCL2/MCP-1 that lacks the murine C-terminal region was also cleaved by meprin α at the N-terminus resulting in significant loss of CCL2/MCP-1 biological activity, whereas meprin β did not affect the biological activity. These studies suggest that meprin α and meprin β may play important roles in regulating the CCL2/MCP-1 chemokine activity during inflammation.
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## 1. Introduction

Monocyte chemotactic protein 1 (CCL2/MCP-1), a chemotactic cytokine, belongs to the CC-type chemokine family and is also known as CCL2 (the chemokine CC motif ligand 2) [1]. CCL2/MCP-1 is involved in the recruitment and trafficking of mononuclear immune cells to inflammation sites [2,3] and plays important roles in the pathogenesis of chronic inflammatory diseases including asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis, and renal inflammatory diseases [1,4–8]. Among the CC chemokines, CCL2/MCP-1 is the most potent chemotactic cytokine [9]. Human CCL2/MCP-1 is comprised of 76 amino acids [10], whereas mouse and rat express CCL2/MCP-1 with an additional 49 amino acids at the C-terminal region [11,12] that aid in increasing the local concentration of CCL2/MCP-1. The monocyte chemoattractant activity of CCL2/MCP-1 is due to its N-terminal domain and the additional 49 amino acids at the C-terminal region are not required for the chemotactic activity [9,13].

Meprins are oligomeric metalloproteinases of the 'astacin' family composed of α and/or β subunits. Meprin α and meprin β are widely distributed in various organs including the kidney, intestines, leukocytes, skin, and bladder, and in a variety of cancer cells [14,15]. The distribution of meprin subunits reveals that they are expressed independently or co-expressed in various organs and cells. For example, both meprin α and meprin β are expressed in the kidney and small intestine whereas only meprin α, but not meprin β, is expressed in the large intestine [16–18]. Also, meprin α and meprin β are expressed in separate layers of the human epidermis [19]. Both meprin α and meprin β are expressed in leukocytes of the lamina propria of the human inflamed bowel [16] and mouse mesenteric lymph nodes [20]. Meprin β is an integral membrane protein anchored to the plasma membrane as a type 1 protein. When meprin α and meprin β subunits are co-expressed, meprin α can either self-associate to form homomeric meprin A or associate with meprin β to form membrane-bound heteromeric meprin A [21,22]. Meprin β subunits can self-associate to form the homodimer meprin B [21]. Meprin α and meprin β are capable of hydrolyzing or processing a large number of substrates including extracellular matrix proteins, cytokines, adherens junction proteins, hormones, bioactive peptides, and cell-surface proteins [15,23,24]. Recent studies have implicated meprins in inflammatory diseases including acute kidney injury, sepsis, urinary tract infections, bladder inflammation, and inflammatory bowel disease (IBD) [15,24–26], but the specific roles of meprins in inflammation are not well understood. Cytokines, including

* Correspondence to: Central Arkansas Veterans Healthcare System, 4300 West 7th Street, 111D, Little Rock, AR, USA.
  E-mail address: kaushalgurp@uams.edu (G.P. Kaushal).

chemokines, are known to regulate inflammatory responses and control immune-cell recruitment in inflammatory processes [27,28]. We have shown that meprin α, meprin β, and heteromeric meprin A purified from the kidney cortices are able to generate biologically active IL-1β from its inactive proform [29,30]. In addition, meprin β was capable of generating biologically active IL-18 from pro-IL-18 [31], whereas both meprin α and meprin β cleaved IL-6 to smaller products that were biologically inactive [32]. These studies suggest involvement of meprins in the processing of cytokines during inflammatory processes. Little information is available, however, on whether chemokines that regulate trafficking of immune cells to the sites of inflammation are targets of meprins. While a previous review reported that meprin α is able to cleave CCL2/MCP-1 [33], the specificity of this cleavage as well as cleavage by other meprin isoforms and the impact of cleaved forms of CCL2/MCP-1 on its biological activity have not been determined. We have examined whether meprin A purified from the kidney cortex or recombinant meprin α and meprin β are capable of cleaving and producing biologically active fragments of CCL2/MCP-1. Furthermore, the specific sites of cleavage in the CCL2/MCP-1 molecule by meprin isoforms were determined.

## 2. Materials and methods

### 2.1. Reagents

Full-length mouse CCL2/MCP-1 (Cat #Sc-4906) and its rabbit polyclonal antibody against CCL2/MCP-1 were purchased from Santa Cruz Biotechnology (Dallas, TX). Recombinant human (Cat #279-MC/CF) and mouse (Cat #479-JE/CF) mature CCL2/MCP-1 (Gln1 through Arg73) and human promeprin α and promeprin β were purchased from R&D Systems (Minneapolis, MN). Both human and mouse CCL2/MCP1 from R&D Systems were used in the cell migration assays and time-course digestion with meprin α and meprin β. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

### 2.2. Cell culture

Human embryonic kidney-293 (HEK-293) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA) were grown in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1x Antibiotic-Antimycotic at 37 °C in a humidified atmosphere containing 5% $CO_2$. Human monocytic (THP-1) cells were grown in Roswell Park Memorial Institute culture medium (RPMI-1640), including 10% FBS, 1x Antibiotic-Antimycotic, 10 mM HEPES, 1 mM Na-pyruvate and 50 μM 2-mercaptoethanol at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cell culture reagents were from Invitrogen (Carlsbad, CA).

### 2.3. Expression plasmid constructs

The plasmid pMepβΔTM1puro3GW for meprin β expression was constructed and analyzed as described in our previous studies [29]. His$_6$-tagged rat meprin α-transfected HEK-293 cells were kindly provided by Dr. Judith Bond [34].

### 2.4. Expression and purification of meprin α

The recombinant His$_6$-tagged rat meprin α was expressed in HEK-293 cells, purified, and characterized as described in our previous studies [30].

### 2.5. Expression and purification of meprin β

The meprin β expression construct, pMepβΔTM1puro3GW, was expressed in HEK-293 cells, purified, and characterized as described in our previous studies [29]. This expression plasmid expresses meprin β that lacks a transmembrane domain.

### 2.6. Purification of meprin A from the rat kidney cortex

Meprin A (meprin αβ) was purified from rat kidney cortices essentially as we described previously [23].

### 2.7. Proteolytic digestion of full-length CCL2/MCP-1

Recombinant full-length mouse CCL2/MCP-1 (1 μg/20 μL, ∼3.6 μM) was digested with activated promeprin α, promeprin βΔ, or meprin A (0.1 μg/20 μL, ∼59 nM), respectively, at room temperature for 90 min. EDTA (10 mM) was added to inhibit meprin activity. Digested samples were separated on 10% NuPAGE Bis-Tris protein gels (Invitrogen), blotted on polyvinylidene difluoride (PVDF), and probed with a rabbit polyclonal antibody against CCL2/MCP-1. Signals were detected by chemiluminescence (SuperSignal WestPico from Pierce, Rockford, IL) and images were recorded on a Chemidoc XRS Imager (Bio-Rad) using QuantityOne Software.

### 2.8. Sequencing of cleaved CCL2/MCP-1 fragments

N-terminal protein sequencing: recombinant full-length mouse CCL2/MCP-1 (1.5 μg/20 μL, ∼5.4 μM) was digested with activated promeprin α, promeprin βΔ, or Meprin A (0.1 μg/20 μL, ∼59 nM), respectively, at room temperature for 90 min. EDTA (10 mM) was added to inhibit meprin activity. Digested samples were separated on 10% Bis-Tris protein gels (Invitrogen), blotted on PVDF, and visualized with Coomassie CBB-R250. Bands of processed CCL2/MCP-1 were excised and submitted to Harvard MicroChem (Dr. William Lane, Cambridge, MA) for N-terminal amino acid analysis using a PE/ABD Procise 494 HT Protein Sequencing System.

C-terminal protein sequencing: Recombinant full-length mouse CCL2/MCP-1 (2 μg/30 μL, ∼4.8 μM) was digested with activated promeprin α, promeprin βΔ, or Meprin A (0.2 μg/30 μL, ∼78.4 nM), respectively, at room temperature for 90 min. EDTA (10 mM) was added to inhibit meprin activity. Digested samples were separated on 10% Bis-Tris protein gels and visualized with Coomassie CBB-R250. The CCL2/MCP-1 fragment produced with activated promeprin βΔ was excised from the gel and submitted for C-terminal amino-acid sequencing at Harvard MicroChem by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer.

### 2.9. Biological activity assay of cleaved CCL2/MCP-1 fragments

Recombinant mature mouse or human CCL2/MCP-1 (1.0 μM) was digested with activated promeprin α (0.10 μM) or promeprin β (0.10 μM) at room temperature for 90 min. Actinonin (50 μM) was added to block meprin activity and the digested CCL2/MCP-1 solutions were stored at 4 °C overnight. Aliquots were analyzed by western blot on 15% SDS-PAGE and PVDF membranes probed with rabbit polyclonal antibody against CCL2/MCP-1. Signals were detected by chemiluminescence (SuperSignal WestPico from Pierce, Rockford, IL) and images recorded on conventional autoradiography film.

The chemotactic migration assay was performed in triplicate in 8.0 μm transwell plates (Corning, New York, NY). THP-1 cells were seeded at $3 \times 10^6$ cells/mL in RPMI medium containing 0.2% BSA in

the upper chamber. Digested or undigested mouse or human CCL2/MCP-1 (5.0 nM) in RPMI medium/0.2% BSA was placed in the bottom chamber. Cells were allowed to migrate at 37 °C in a humidified $CO_2$-incubator for 4 h. A cell-counting kit-8 reagent (Dojindo Molecular Technologies, Rockville, MD) was added to the bottom chamber and incubated for 4 h at 37 °C in a $CO_2$-incubator and the absorbance read at 450 nm in a SpectramaxM5 microplate reader (Molecular Devices, Sunnyvale, CA). The values were obtained from four independent experiments.

## 3. Results

### 3.1. Cleavage of mouse CCL2/MCP-1 by meprin α, meprin β, and heteromeric meprin A and identification of the cleaved sites

Meprins were purified and characterized as described in our previous studies [29,30]. We tested whether meprins are able to cleave CCL2/MCP-1. Meprins were first activated by limited trypsin digestion that was terminated by the addition of a soybean trypsin inhibitor (SBTI). When activated recombinant meprin α, meprin β, or heteromeric meprin A purified from kidney were incubated with mouse full-length CCL2/MCP-1, specific cleaved products were detected by western blot (Fig. 1). N-terminal amino acid sequencing of the cleaved products revealed that meprin α and meprin A cleaved between $Asn^6$ and $Ala^7$ whereas meprin β was unable to cleave the N-terminus of CCL2/MCP-1. Meprin β cleaved between $Ser^{74}$ and $Glu^{75}$ in the C-terminus to result in truncated CCL2/MCP-1 with 74 amino acids (Fig. 2). The cleavage sites at the C-terminal region shown for meprin α and meprin A are based on their specificities [35]. These studies showed that meprin α and meprin A but not meprin β are able to process the N-terminus of mouse CCL2/MCP-1 (Fig. 2). Since the chemoattractant activity of CCL2/MCP-1 is known to reside in the N-terminus [13], it was of interest to determine whether processing of the N-terminus by meprins affects the chemotactic activity.
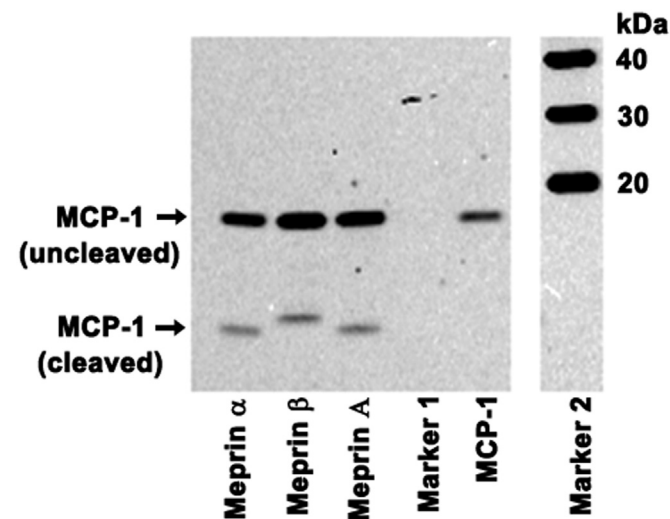
### 3.2. Processing of chemotactic N-terminus of mouse CCL2/MCP-1 by meprin α and meprin β and determination of chemotactic activity of the processed products

Recombinant mature mouse CCL2/MCP-1 (Gln1 through Arg73) was incubated with activated meprin α and meprin β for various time periods and the time-dependent proteolytic processing of mouse CCL2/MCP-1 by meprin α and meprin β was detected by western blot (Fig. 3).
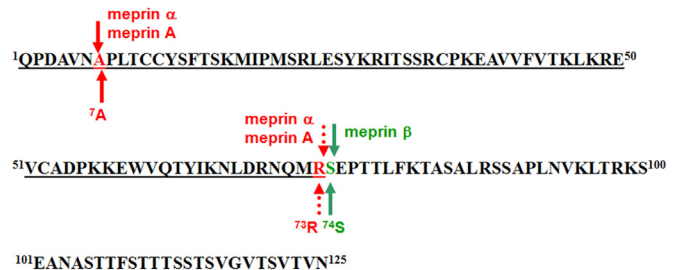
**Fig. 2.** Sites of enzyme cleavage by meprins in the full-length mature murine CCL2/MCP-1 sequence. Sequence of full-length mature mouse CCL2/MCP-1 showing the sites of enzyme cleavage for recombinant human meprin βΔ, recombinant rat meprin α, and meprin A purified from rat kidney. Dashed arrows indicate the putative C-terminal amino acid of the fragment produced with meprin α and meprin A. The N-termini were determined by Edman degradation, the C-terminus produced by meprin α was determined by LC/MS/MS. Sequence of truncated mature mouse CCL2/MCP-1 is underlined.
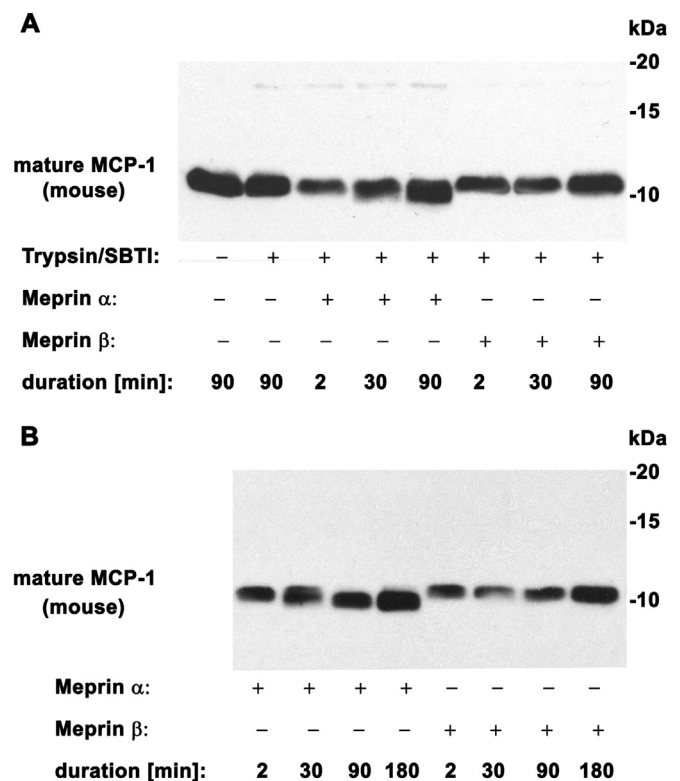
**Fig. 3.** Time course of CCL2/MCP-1 cleavage by meprins. **Panel A.** Recombinant mature mouse CCL2/MCP-1 (1.0 μM) was digested with activated human promeprin α (0.10 μM) or human promeprin β (0.10 μM) respectively at 37 °C for 2, 30, and 90 min. Actinonin (50 μM) was added to block meprin activity. Aliquots (100 ng) were separated on 15% SDS-PAGE, blotted on PVDF membranes and blots probed with rabbit polyclonal antibody against CCL2/MCP-1. Incubation of CCL2/MCP-1 with trypsin/SBTI was used to demonstrate complete deactivation of trypsin by SBTI used in the activation of promeprins. **Panel B.** Recombinant mature mouse CCL2/MCP-1 (1.0 μM) was digested with activated human promeprin α (0.10 μM) or human promeprin β (0.10 μM) respectively at 37 °C for 2, 30, 90, and 180 min and subjected to SDS-PAGE as described for Panel A.

**Fig. 1.** Cleavage of mouse CCL2/MCP-1 by meprins. Activated recombinant rat meprin α, recombinant human meprinβΔ, and meprin A from rat kidney membranes (100 ng) were incubated with full-length mature mouse CCL2/MCP-1 (100 ng) at 37 °C for 90 min. Samples (50 ng) were separated by 10% SDS-PAGE and products detected on western blots with an anti-CCL2/MCP-1 rabbit polyclonal antibody (1:500 dilution). Untreated CCL2/MCP-1 (10 ng) was used as a reference (lane 5). Kaleidoscope Marker (Bio-Rad), **Marker 1** (not visible, lane 4) was used to monitor samples during gel run. Sizes of Magic Mark XP (Invitrogen) **Marker 2** protein standards (separate lane) are indicated at right. Separate lane as shown was obtained from the same western blot.

The processing of CCL2/MCP-1 by meprin α as detected by the clear band shift which was evident after 90 min of incubation (Fig. 3A and B), whereas the processing of CCL2/MCP-1 by meprin β remained unchanged even at 180 min of incubation. Chemotactic activity of the cleaved products generated on incubation of activated meprin α and meprin β was determined upon incubation of recombinant truncated mouse CCL2/MCP-1 (Fig. 4A). As shown in the figure, a clear band-shift of the product is produced by meprin α. The chemotactic activity was also significantly reduced with the cleaved product generated by meprin α, which was lower than that produced by meprin β (Fig. 4B left panel). Since the N-terminus of mouse CCL2/MCP-1 is similar and of the same size of human CCL2/MCP-2, we examined the effect of cleavage of human CCL2/MCP-2 by meprins on the chemotactic activity. As shown in Fig. 4B, right panel, the chemotactic activity of the cleaved human CCL2/MCP-2 upon incubation with meprin α was markedly reduced.
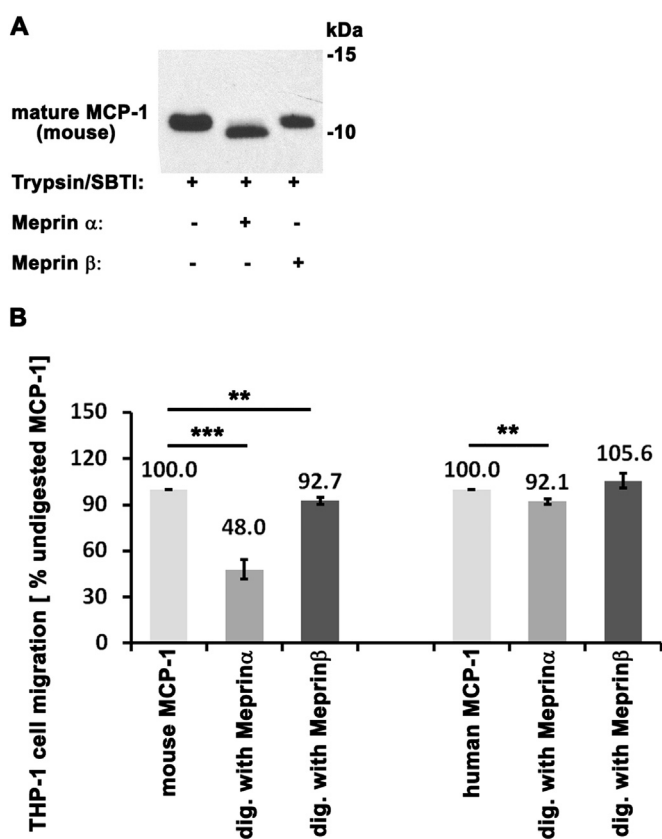


Fig. 4. Biological activity assay of cleaved CCL2/MCP-1 fragments. **Panel A.** Western blot of cleaved CCL2/MCP-1 fragments used in CCL2/MCP-1 migration assay. Recombinant mature mouse CCL2/MCP-1 was digested with activated human pro-meprin α and activated promeprin β at 37 °C for 90 min. Aliquots of the individual digests were removed and used for the migration assay and aliquots (50 ng) of the remaining samples analyzed by western blot as described in Materials and Methods. A representative western blot is shown. **Panel B.** The chemotactic migration assays were performed in triplicate in 8.0 μm transwell plates. THP-1 cells were seeded at $3 \times 10^6$ cells/mL in RPMI medium containing 0.2% BSA in the upper chamber. Digested or undigested mouse or human CCL2/MCP-1 (5.0 nM) in RPMI medium/0.2% BSA was placed in the bottom chamber. Cells were allowed to migrate at 37 °C in a humidified $CO_2$-incubator for 4 h. Cell counting kit-8 reagent was added to the bottom chamber and plates incubated for 4 h at 37 °C in the $CO_2$-incubator. Cell migration was monitored by reading absorbance at 450 nm in a SpectramaxM5 microplate reader. Cell migration obtained with digested CCL2/MCP-1 was normalized to migration obtained with untreated CCL2/MCP-1. The values displayed represent averages ± SEM of four independent experiments (separate cleavage reactions). Results of Student's T-test are shown with **$P < 0.01$ and ***$P < 0.001$.

## 4. Discussion

Meprins are able to cleave a wide variety of substrates including basement membrane proteins, cytokines, adherens junction proteins, growth factors, protein kinases, bioactive peptides, and cell-surface proteins [15,23,24,37–39]. However, both subunits are known to show marked differences in cleavage specificities of the various substrates [15,35]. The present study demonstrates that recombinant human meprin α and meprin A purified from rat kidneys are capable of processing both the N- and C-terminal regions of murine CCL2/MCP-1. In contrast, meprin β was able to cleave the C-terminal region of full-length mouse MCP-1 but was unable to process the N-terminus. Only the N-terminal region of the full-length mouse CCL2/MCP-1 that is homologous to the full-length human CCL2/MCP-1 exhibits chemotactic activity [9,13]. Our studies show that meprin α and meprin A but not meprin β eliminated the first six amino acids at the N-terminus and the resulting truncated CCL2/MCP-1 variant exhibited significantly reduced chemotactic activity. In addition, we showed that human CCL2/MCP-1 activity is less affected by meprin α cleavage than mouse CCL2/MCP-1 activity but the loss in chemotactic activity was significant. We attribute this effect to differences of the amino-acid sequences in regions of human and mouse CCL2/MCP-1 other than the N-terminus regions. Previous studies by site-directed mutagenesis have shown that deletion of amino acid residues at the N-terminus of CCL2/MCP-1 results in loss of chemotactic activity [9,40]. This specificity of meprins toward CCL2/MCP-1 has not been described previously.

Multiple extracellular metalloproteinases are known to cleave the N-terminal region of CCL2/MCP-1 that renders CCL2/MCP-1 inactive [41]. For example, MMP-1 and MMP-3 [42] and MMP-2 and MMP-9 [43] cleave the first four amino acids at the N-terminus and render CCL2/MCP-1 inactive for chemotaxis. Our studies showed that cleavage of the first six amino acids at the N-terminus by meprin α and meprin A also reduced chemotactic activity of CCL2/MCP-1. Thus, our studies support the notion that deletion of amino acids at the N-terminal region results in the loss of chemotactic activity. The cleaved CCL2/MCP-1 can also act as a potent antagonist to macrophage chemotaxis. The truncated CCL2/MCP-1 generated by increased MMP-2 activity functioned as an antagonist for CCR-2 signaling [44]. Considering these reports it is quite tempting to conclude that the cleaved CCL2 (that lacks the N-terminal peptide) produced by meprin A or meprin α is responsible for the loss of chemotactic activity. However, at present we do not know whether released N-terminal peptide is able to contribute to the loss of chemotactic activity. Plasmin is known to process mouse CCL2/MCP-1 but instead of N-terminal processing it cleaves the C-terminal region [45], much like the C-terminal cleavage we observed in this study with meprin β. The chemotactic activity of the plasmin-cleaved mouse CCL2/MCP-1 was similar to that of human CCL2/MCP-1 [46,47]. Our studies showed that meprin β cleaved mouse CCL2/MCP-1 resulting in CCL2/CCL2/MCP-1 activity comparable to human CCL2/MCP-1 activity. Therefore, cleavage of mouse CCL2/MCP-1 with meprin β is similar to plasmin-mediated cleavage of CCL2/MCP-1 that maintains chemokine activity.

Meprin A is a major metalloproteinase of the brush-border membranes of the kidney, and meprin α and meprin β are abundantly expressed in the intestines, skin, macrophages, and several cancer cells. Previous studies showed that meprins play important roles in inflammatory diseases including intestinal inflammation, acute kidney injury, fibrosis, and cancer [15,24]. Meprin α-KO mice in a model of DSS (dextran sodium sulfate)-induced colitis exhibited more severe inflammation and intestinal injury than observed with wild-type mice, whereas meprin β-KO mice in this model had less inflammation and lower levels of pro-inflammatory IL-18 cytokine

[31]. These studies suggest that meprin isoforms have opposing effects, most likely due to marked differences in their cleavage specificities to the cytokines. Our studies showed that meprin A and meprin α, but not meprin β, inactivate CCL2/MCP-1 and that this regulation may have profound effects on inflammatory diseases including acute kidney injury, cancer, and chronic inflammation of the gastrointestinal tract.

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## Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.019.

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